Universitat Autònoma de Barcelona

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PhD Thesis

Bellaterra (Barcelona), 2021

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Doctoral Thesis presented by **Viviane Saporiti** to obtain the Doctoral degree under the program of Animal Medicine and Health at the Faculty of Veterinary Medicine from *Universitat Autònoma de Barcelona*, under the supervision of **Joaquim Segalés** and **Marina Sibila**.

Bellaterra (Barcelona), 5th June, 2021.

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The research studies done in the doctoral thesis work entitled **"Porcine circovirus 3 (PCV-3) infection and association with disease in swine"**, presented by **Viviane Saporiti** to obtain the Doctoral Degree in *Medicina i Sanitat Animals* was done under their supervision, and authorize its presentation to be evaluated by the corresponding commission.

We signed this certificate for the records in Bellaterra (Barcelona), 5th June 2021.

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Certifican que:

Los trabajos de investigación desarrollados en la memoria de tesis doctoral **"Porcine circovirus 3 (PCV-3) infection and association with disease in swine"**, presentados por la licenciada **Viviane Saporiti** para la obtención del grado de Doctora en *Medicina i Sanitat Animal* se ha realizado bajo la dirección, y autorizan su presentación a fin de ser evaluada por la comisión correspondiente.

Y porque así conste y tenga los efectos que correspondan, firman el presente certificado.
Bellaterra (Barcelona), 5 de Junio de 2021.

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ACKNOWLEDGEMENTS

First of all, I would like to thank God for being my daily strength and the guide of my life.

Secondly, I would like to thank my heroes, my mom and dad, for being the outstanding people that they are, and for everything they have sacrificed for our family. For their endless efforts to support my dreams, above all, for believing in me even in times I did not believe myself. I cannot imagine how many dreams of their own they gave up, for me to live my own. Every night my heart is tightly squeezed by the feeling of them giving their all to me, I pray for God that He grants them in double everything they have done for me. I thank my mom and dad for being the first ones to support the calculated craziness of leaving my comfort zone and the "apparent safe future" and start the journey overseas in pursuing my Doctorate and thereafter open my horizons. For being on my side and every month support me financially, with and without COVID-19 and with an extremely volatile exchange rate. Finally, also thank them for being emotionally strong and for teaching me to become a little stronger every day.

I wish this baby in my womb loves me and admires me the same way I love and admire both of my parents, because I cannot imagine a stronger feeling. I thank my brother Bruno, who is an example of determination and resilience and together with my sister-in-law Val, always blindly supporting and encouraging me in all my decisions.

From the bottom of my heart, I would like to thank the one who is my new family, my beloved husband and Antoni's father. Thank you for showing me unconditional love every day and for joining me on this adventure, leaving your dreams and plans aside to live mines. For being my daily encourager and showing me with attitudes how to be a kinder and better person in every detail of life. Thanks for being this safe heaven, for fighting so much and in so many ways for our family, our dreams, our long-awaited miracle (our son) and for making our home the best place in the world to return after long days of work.

In a special way I would like to thank my mentors Quim and Marina. I believe that for someone's professional growth, having mentors is perhaps the best path to grow and ultimately to succeed. I am very grateful that I did not have just two bosses, during this PhD, but two mentors, and mentors are those who we admire and get inspired by. Quim, thank you so much for supporting me the way you did and for fostering my growth intellectually, and as a person. Your way of leading and encouraging people is simply amazing. I feel very blessed to have had the opportunity to work with you and for being able to witness your

outstanding work and learn from it. Marina, you were an amazing present that came along with the change of my project's Thesis. You are an example and an inspiration of a successful woman, and an amazing researcher that I'm proud to follow the steps. Thank you for the many lessons you though me and for showing me that every single detail matters. With your kind, sweet and encouraging way, it has been essential for me to reach my big and small achievements. The both of you together is simply the best team I could ever had during these years, thank you both so much for everything. These were the most transforming years of my life.

Flor, thank you for all the patience you have had with me and that bioinformatics requires to be taught. You introduced me to a new world and taught me things that I imagined I would never like. Even though I'm still crawling, you have opened my eyes to understand and see things differently. Thank you for being part of this thesis, for your effort and for teaching me in each of these studies.

I also would like to thank my former advisors, who in such a short time have taught me so much. Júlia, it was only a few months on the same team, but everything you did for me has had a profound impact in my path. Maybe I wouldn't have finished my PhD if it weren't for the opportunity you gave me by hiring me as a technician at that time. Not only for the financial opportunity but specially for the warm and kind way you have received me, all the help you provided with the bureaucracy and the emotional support I much needed. Albert, I have no words to thank you for the teachings and the growth I had in the 6 months working with you. Between tears and lots of mistakes during those months, you showed me that I am capable of much more than I thought I would and that we can always learn new things, no matter how challenging it may look. You both have my eternal gratitude and admiration.

I extend my gratitude to my laboratory family; Eva and Anna. I would like to thank you both for so many teachings and moments shared, it was an honor for me. Thank you for teaching me with so much care and affection which went beyond laboratory techniques, you both made the work routine light and pleasant. I also thank the entire CReSA team, which makes this a very special, with a family feeling and an extremely pleasant workplace.

My thanks to the partner companies and all veterinarians involved, which efforts and collaboration were essential to make these studies a reality.

Finally, thank you all my fellows "becarios". Thanks to each one of you for being a part of these wonderful journey and for sharing so many good moments. The best memories

of these years are with you guys. I have learned from each one of you, laughed with many and cried with some. I am deeply grateful and carrying with me a special mark of each one of you. You all will be forever in my heart and I hope to meet you all whenever it is possible.

Thank you all from the bottom of my heart!!!

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LIST OF ABREVIATIONS

aa	Amino acids
Ab	Antibody
ADV	Aujeszky's disease virus
AFV	Atrophy and fusion of villi
APPV	Atypical porcine pestivirus
BLQ	Below the limit of quantification
bp	Base pairs
Cap	Capsid associated protein
CC	Catarrhal colitis
CE	Catarrhal enteritis
CNS	Central nervous system
CRL	Crown-to-rump
CSFV	Classical swine fever virus
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
dpi	Days post-infection
dsDNA	Double stranded DNA
EMCV	Encephalomyocarditis virus
ELISA	Enzyme-Linked Immuno Sorbent Assay
FNP	Fibrinous-necrotizing pleuropneumonia
HEV	Hepatitis E virus
IAV	Influenza A virus
ICTV	International Committee on the Taxonomy of Viruses
IFA	Immunofluorescent assay
IHC	Immunohistochemistry
IP	Interstitial pneumonia
IPMA	Immunoperoxidase monolayer assay
ISH	In situ hybridization
MAb	Monoclonal Antibody
Mhyo	*Mycoplasma hyopneumoniae*
MiCV	Mink circovirus
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
NS	Non-structural protein
nt	Nucleotide
ORF	Open reading frame
P	Pleuritis
PAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PBoV	Porcine bocavirus
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV-1	Porcine circovirus 1
PCV-2	Porcine circovirus 2
PCV-2-RD	PCV-2 reproductive disease
PCV-2-SD	PCV-2 systemic disease

PCV-2-SI	PCV-2 subclinical infection
PCV-3	Porcine circovirus 3
PCV-3-RD	PCV-3 reproductive disease
PCV-3-SD	PCV-3 systemic disease
PCV-4	Porcine circovirus 4
PCVD	Porcine circovirus disease
PDNS	Porcine dermatitis and nephropathy syndrome
PEDV	Porcine epidemic diarrhea vírus
PEV	Porcine enteroviruses
PK-15	Porcine kidney cells
PKV	Porcine kobuvirus
PMWS	Postweaning multisystemic wasting syndrome
PPV	Porcine parvovirus
PRRSV	Porcine respiratory and reproductive syndrome virus
PRV	Pseudorabies virus
PSV	Porcine sapelovirus
qPCR	Real-time quantitative PCR
Rep	Replication associated protein
RNA	Ribonucleic acid
SBP	Suppurative bronchopneumonia
SIV	Swine influenza virus
SNP	Single nucleotide polymorphisms
ssDNA	Single stranded DNA
ST	Swine testicles cells
TTSuV	Torque teno sus virus
UK	United Kingdom
USA	United States of America

ABSTRACT

Porcine circovirus 3 (PCV-3) has been identified in pigs suffering from different disease conditions, as well as in healthy animals. However, the mere detection of the virus does not imply disease causality. Moreover, in most studies, a healthy control group was not included, complicating the interpretation of the potential implication of the virus in the disease development. Thus, this Thesis was aimed to study the potential of PCV-3 as a cause of disease in pigs. To reach this main goal, four studies were conducted.

The first study assessed the potential association of PCV-3 with respiratory or enteric diseases in pigs. Sera of postweaning pigs displaying respiratory (n=129) or digestive (n=126) disorders and of healthy ones (n=60) were analyzed by PCR and, when positive, by a real time quantitative PCR (qPCR). Moreover, selected samples were sequenced and phylogenetically analyzed. PCV-3 was found in 6% in animals showing respiratory (8/129) and digestive (7/126) disorders, and in 7% (4/60) of healthy pigs. The similar percentages of infection among groups indicated that PCV-3 is not a potential cause of respiratory or digestive disorders.

Secondly, the frequency of PCV-3 infection was studied in sera of primiparous (n=57) and multiparous (n=64) sows at two time points, and in tissues (brain and lung) from their respective stillborn or mummified fetuses (n=255) from three farms without reproductive problems. Samples were tested by PCR and, when positive, by a qPCR; selected samples were sequenced and phylogenetically analyzed. All sera from multiparous sows were negative, while 19/57 (33%) sera from primiparous sows were positive. Fetuses showed 33% (86/255) of PCR positivity; however, the frequency of viral detection in fetuses from primiparous sows (73/91, 80%) was significantly higher than that from multiparous ones (13/164, 8%). This study demonstrated that PCV-3 can cause intrauterine infections in absence of overt reproductive disorders.

In a third study, the frequency of PCV-3 in cases of overt reproductive problems and the possible association with the lesions observed in aborted fetuses were evaluated. Thus, 53 reproductive cases (tissue pools) were analyzed by qPCR. Samples with high PCV-3 load were further tested by *in situ* hybridization (ISH), sequenced and phylogenetically analyzed. PCV-3 DNA was detected in 18/53 (34%) cases by qPCR and in 4 out of the 6 tested by ISH. Other viruses able to cause reproductive diseases (Porcine reproductive and respiratory syndrome virus [PRRSV], Porcine parvovirus 1 [PPV1] and Porcine circovirus 2 [PCV-2]) were also investigated. PRRSV and PCV-2 were found in 4 and 5 cases, respectively. The

higher PCV-3 loads detected in some cases of reproductive disorders and their association with lesions point out this virus as a potential agent able to cause reproductive failure.

In the phylogenetical analyses performed in these three studies, all whole genome and ORF-2 PCV-3 sequences obtained were classified as PCV-3a, being closely related with regards nucleotide identity.

In the fourth study, samples of four post-weaning pigs displaying wasting (negative to PCV-2 and PRRSV), and a multisystemic inflammatory condition, were analyzed by a PCV-3 ISH. Two non-affected animals from the same farm were also tested. PCV-3 was found within histologic lesions in multiple tissues of the four diseased animals, while it was not found in the two non-affected animals. The present case report further suggests PCV-3 as a potential infectious agent able to cause disease in post-weaning pigs.

In conclusion, this Thesis generated relevant knowledge on clinical, pathological and virological data of PCV-3 infection. These data further support the potential pathogenicity of this virus and, thus, highlighting the need to establish diagnostic criteria for the reproductive and pre-/post-weaning disorders of swine.

RESUMEN

El circovirus porcino 3 (PCV-3) se ha identificado en cerdos afectados por diversas condiciones clínico-patológicas, así como en animales sanos. Sin embargo, la mera detección del virus no implica causalidad en la enfermedad. Además, muchos de los estudios publicados no incluyen un grupo de animales sanos como control, dificultando la interpretación de la posible implicación del virus en la enfermedad. Esta Tesis tuvo como objetivo estudiar el potencial de PCV-3 como causa de enfermedad. Para ello, se realizaron cuatro estudios.

En el primer estudio se evaluó la posible asociación de PCV-3 con desórdenes respiratorios o entéricos. Se analizaron sueros de cerdos post-destete que presentaban trastornos respiratorios (n=129) o digestivos (n=126) así como de animales sanos (n=60). Las muestras se analizaron mediante PCR y, en los positivos, mediante una qPCR. Parte de las muestras positivas, se secuenciaron y analizaron filogenéticamente. PCV-3 se detectó en un 6% tanto en animales con trastornos respiratorios (8/129) como digestivos (7/126), y en un 7% (4/60) en los animales sanos. La similitud en los porcentajes de detección indicó que PCV-3 no parece una posible causa de enfermedad respiratoria o digestiva.

En segundo lugar, se estudió la frecuencia de PCV-3 en sueros de cerdas primíparas (n=57) y multíparas (n=64) en dos momentos de la gestación, y en tejidos (cerebro y pulmón) de sus respectivos fetos mortinatos o momificados (n=255) procedentes de tres granjas sin problemas reproductivos. Las muestras se analizaron mediante PCR y, en los positivos, mediante una qPCR. Parte de las muestras positivas se secuenciaron y analizaron filogenéticamente. Mientras que todos los sueros de cerdas multíparas fueron negativos, un 33% (19/57) de sueros de cerdas primíparas fueron positivos. Los fetos mostraron un 33% (86/255) de positividad. La frecuencia de detección en fetos de cerdas primíparas (73/91, 80%) fue significativamente mayor que la de multíparas (13/164, 8%). Este estudio demostró que PCV-3 puede causar infecciones intrauterinas en ausencia de trastornos reproductivos evidentes.

En un tercer estudio, se evaluó la frecuencia de detección de PCV-3 en casos de problemas reproductivos evidentes y la posible asociación con lesiones observadas en fetos abortados. Se analizaron 53 casos reproductivos (mezcla de tejidos) mediante qPCR. Las muestras con alta carga vírica se analizaron mediante hibridación *in situ* (ISH) y se secuenciaron y analizaron filogenéticamente. PCV-3 se detectó en 18/53 (34%) casos mediante qPCR y en 4 de los 6 analizados por ISH. También se investigaron otros virus

causantes de enfermedades reproductivas (virus del síndrome respiratorio y reproductivo porcino [PRRSV], parvovirus porcino 1 [PPV1] y circovirus porcino 2 [PCV-2]). PRRSV y PCV-2 se detectaron en 4 y 5 casos, respectivamente. Los casos con mayor carga vírica de PCV-3 y su asociación con las lesiones sugieren al virus como potencial causante potencial de problemas reproductivos

Los análisis filogenéticos realizados en estos tres primeros estudios clasificaron a las secuencias del genoma entero y del ORF-2 obtenidas como PCV3a, mostrando una elevada identidad nucleotídica.

Por último, se testaron muestras de tejidos de cuatro cerdos post-destete, clínicamente afectados por desmedro (negativos a PCV-2 y PRRSV) y con inflamación multisistémica, mediante una ISH especifica de PCV-3. Además, también se incluyeron dos animales de la misma granja no afectados clínicamente. El virus se detectó en histológicas observadas en múltiples tejidos de los cuatro animales enfermos, pero no en los dos animales sanos. Estos resultados, apuntan que PCV-3 pueda causar enfermedad en cerdos post-destete.

En conclusión, en esta Tesis se han generado conocimientos relevantes sobre datos clínicos, patológicos y virológicos de la infección por PCV-3. Estos datos sugieren que el virus puede causar enfermedad en cerdos y resaltan la necesidad de establecer criterios diagnósticos para los trastornos reproductivos y pre-/post-destete.

RESUM

El circovirus porcí 3 (PCV-3) s'ha identificat en porcs afectats per diversos problemes clínics, així com en animals sans. No obstant, la mera detecció de virus no implica causalitat en la malaltia. A més, molts dels estudis publicats no inclouen un grup d'animals sans com a control, dificultant així la interpretació de la possible implicació del virus en el desenvolupament de la malaltia. L' objectiu d'aquesta Tesi va ser estudiar el potencial de PCV-3 com a causa de malaltia a través de quatre estudis.

En el primer estudi es va avaluar la possible associació de PCV-3 amb malalties respiratòries o entèriques. Es van analitzar sèrums de porcs post-deslletament que patien trastorns respiratoris (n=129) o digestius (n=126) i d'animals sans (n=60). Les mostres es van analitzar mitjançant PCR i els positius mitjançant una qPCR. Part de les mostres positives, es van seqüenciar i analitzar filogenèticament. PCV-3 es va detectar en un 6% tant en animals amb trastorns respiratoris (8/129) com digestius (7/126) i en un 7% (4/60) en els animals sans. grups Aquest resultats indiquen que PCV-3 no és una possible causa de trastorns respiratoris o digestius.

En segon lloc, es va estudiar la freqüència de PCV-3 a sèrums de truges primípares (n=57) i multípares (n=64) en dos moments de la gestació, i en teixits (cervell i pulmó) dels seus respectius fetus nascuts morts o momificats (n=255) procedents de tres granges sense problemes reproductius. Les mostres es van analitzar mitjançant PCR i els positius mitjançant una qPCR. Part de les mostres positives es van seqüenciar i analitzar filogenèticament. Mentre que tots els sèrums de truges multípares van ser negatius, un 33% (19/57) dels sèrums de truges primípares van ser positius. Els fetus van mostrar un 33% (86/255) de positivitat. La freqüència en fetus de truges primípares (73/91, 80%) va ser significativament més gran que la de multípares (13/164, 8%). Aquests resultats indiquen que PCV-3 pot causar infeccions intrauterines en absència de trastorns reproductius evidents.

En un tercer estudi, s'avaluà la freqüència de detecció en casos de problemes reproductius evidents i la possible associació amb lesions observades en fetus avortats. Es van analitzar 53 casos reproductius (mescla de teixits) mitjançant qPCR. Les mostres amb alta càrrega de PCV-3 es van analitzar mitjançant hibridació *in situ* (ISH) i es van seqüenciar i analitzar filogenèticament. PCV-3 es va detectar en 18/53 (34%) casos mitjançant qPCR i en 4 dels 6 analitzats per ISH. També es van buscar altres virus causants de malalties reproductives (virus de la síndrome respiratòria i reproductiva porcina [PRRSV], parvovirus porcí 1 [PPV1] i circovirus porcí 2 [PCV-2]). PRRSV i PCV-2 es van detectar en 4 i 5 casos,

respectivament. L'elevada càrrega vírica de PCV-3 i l'associació amb les lesions observades suggereixen a aquest virus com a un potencial causant de problemes reproductius.

L'anàlisi filogenètic realitzat en aquets tres estudis va classificar les seqüencies obtingudes del genoma sencer o de l'ORF2 com a PCV-3a.

Per últim es van analitzar mitjançant una ISH mostres de quatre porcs post-deslletament clínicament afectats per aprimament (negatius a PCV-2 i PRRSV) i amb inflamació multisistèmica. També es van incloure dos animals de la mateixa granja no afectats. PCV-3 es va detectar en les lesions histològiques en múltiples teixits dels quatre animals malalts, però no en els dos animals sans. Aquests resultats, apunten que PCV-3 pugui causar malaltia en porcs post-deslletament.

En conclusió, en aquesta Tesi s'han generat coneixements rellevants sobre dades clíniques, patològiques i virològiques de la infecció per PCV-3. Aquestes dades suggereixen que el PCV-3 pot causar malaltia en porcs i ressalten la necessitat d'establir criteris de diagnòstic per als trastorns reproductius i pre-/post-deslletament.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Porcine circoviruses (PCVs)

Porcine circoviruses (PCVs) are single stranded DNA (ssDNA) viruses, belonging to the *Circoviridae* family (Tischer et al., 1982). Since 2016, this family is divided in two different genera, namely Cyclovirus and Circovirus (Rosario et al., 2017).

The Circovirus genus comprises viruses that have been detected in different vertebrate species (Lukert et al., 1995; Lőrinez et al., 2010; Lőrinez et al., 2012; Li et al. 2013; Rosario et al., 2017) and some of them are able to cause fatal diseases, such as Beak and feather disease virus (BFDV) in birds and Porcine circovirus 2 (PCV-2) in pigs (Todd, 2000). So far, the International Committee of Taxonomy of Viruses (ICTV) describes three species able to infect pigs within the Circovirus genus: PCV-1, PCV-2 and PCV-3 (Rosario et al., 2017). Even though a fourth member, PCV-4, was recently proposed, it has been only reported in China and in South Korea (Zhang et al., 2019; Nguyen et al., 2021) and it is not yet included in ICTV reports. Table 1.1 shows the main characteristics of all four PCVs described so far.

PCVs are small, non-enveloped viruses with icosahedral symmetry (Ritchie et al., 1989). The diameter of the virions can range from 15 to 25 nm (Todd et al., 1991; Meehan et al., 1998; Crowther et al., 2003; Rosario et al., 2017). PCV genomes are circular and the size ranges from 1,758 to 1,760 nucleotides (nt) for PCV-1 (Fenaux et al., 2003; Meehan et al., 1997), from 1,766 to 1,769 nt for PCV-2 (Fenaux et al.,2000; Guo et al., 2010), and from 1,999 to 2,001 nt for PCV-3 (Fux et al., 2018; Ha et al., 2018). The few information available on PCV-4 reports a genome of 1,770 nt in length (Zhang et al., 2019).

PCV genomes have an ambisense organization and display two major open reading frames (ORFs) arranged in the different strands of the double-stranded DNA (dsDNA) replicative form (Rosario et al., 2017). Although ORF1 and ORF2 are present in all PCVs, other ORFs have been predicted in each PCV species. In PCV-1 further five ORFs are predicted to encode other proteins (Mankertz et al., 1998; Meehan et al., 1997). For PCV-2, nine more ORFs are predicted but only ORF3 and ORF4 are well-characterized and known to encode for functional proteins (Hamel et al., 1998; Gao et al., 2014). In PCV-3, ORF3 is also predicted to codify for a protein, but its function is not yet characterized (Phan et al., 2016; Palinski et al., 2017). For PCV-4, ten other ORFs have also been predicted but no information is available about any of them (Zhang et al., 2019).

Table 1.1. Main characteristics of Porcine circoviruses (adapted from Opriessnig et al., 2020)

Parameters	**PCV-1**	**PCV-2**	**PCV-3**	**PCV-4**
Year of discovery	1974	1997	2016	2019
Earliest detection	1974	1962	1993	2018
First viral isolation	1974	1997	2020	NA
Genome size (nt)	1758-1760	1766-1769	1999-2001	1770
Distribution	Worldwide	Worldwide	Worldwide	China and South Korea
Prevalence	Low	High	High	NA
Serotypes	One	One	One	NA
Genotypes	Non-described	Nine from “a” to “i”	One	NA
Pathology/ies reported (in pigs) in the field	None	Reproductive, Systemic, PDNS	Reproductive, Respiratory, Systemic, PDNS, Neurological	Respiratory, Enteric, PDNS
Pathology/ies reported (in pigs) under experimental conditions	None	Reproductive, Systemic	Systemic (very mild), PDNS-like	None
Types of vaccines	None	Multiple (inactivated, chimeric and subunit)	None	None

NA: Non-available information; PDNS: porcine dermatitis and nephropathy syndrome.

ORF1 is located on the positive sense strand of the virus, encodes for the replication-associated proteins (Rep and Rep'), and is the most conserved region (Mankertz et al., 2004). The ORF2 is in the opposite strand and encodes for the capsid protein (Cap), considered the most variable and immunogenic region of the PCVs (Nawagitgul et al., 2002; Grierson et al., 2004). ORF3 encodes for a third protein with apoptotic capacity in PCV-1 and PCV-2 (Liu et al., 2005), but still not well-characterized in PCV-3 (Palinski et al., 2017). ORF4 has only been well described in PCV-2, located in the negative strand, and codifies for an anti-apoptotic protein (He et al., 2013; Gao et al., 2014; Lv et al., 2016).

Phylogenetic analyses indicate amino acid (aa) similarity regarding Cap gene of 67% between PCV-1 and PCV-2 (Meehan et al., 1998), 24% between PCV-1 and PCV-3 (Phan et al., 2016), and around 26-37% between PCV-2 and PCV-3 (Phan et al., 2016; Palinski et al., 2017). The Cap gene from PCV-4 showed 43.1%, 45.0% and 24.5% of aa similarity when compared to PCV-1, PCV-2 and PCV-3, respectively (Zhang et al., 2019). Regarding the identity of Rep gene, PCV-1 and PCV-2 showed 86% of aa similarity between them (Meehan et al., 1998), while between PCV-2 and PCV-3 was around 48% (Phan et al., 2016). PCV-4 showed 48.1%, 47.2% and 51.4% aa identity within Rep gene of PCV-1, PCV-2 and PCV-3, respectively (Zhang et al., 2019; Tian et al., 2020).

1.2 Porcine circovirus 1

In 1974, a small virus was described persistently infecting the CCL-33 porcine Kidney (PK-15) cell line with no evidence of cytopathic effect (Tischer et al., 1974). The origin of the infection was suspected to be a contamination from a serum or swine tissue used in the cell culture (Dulac and Afshar, 1989). Firstly, the virus genome was thought to be RNA (Tischer et al., 1974), but few years later the agent was confirmed to be a circular ssDNA and named as PCV (Tischer et al., 1982), the currently named PCV-1. *In vitro* studies subsequently showed the capacity of PCV-1 to grow in Vero cells (Allan et al., 1994), besides the PK-15 ones (Tischer et al., 1974).

PCV-1 is considered able to infect pigs at any age, including the possibility of vertical transmission. In fact, the virus has been found in milk from sows (Shibata et al., 2006) and also in stillborn piglets displaying congenital tremors (Allan et al., 1995; Stevenson et al., 2001; Choi et al., 2002). However, its wide distribution based on antibody tests in animals without clinical signs (Tischer et al., 1986; Finsterbusch and Mankertz, 2009; Beach et al., 2010) suggested that PCV-1 infections are probably subclinical.

Experimental infection in pigs demonstrated that PCV-1 was not able to generate lesions in the animal tissues, although it was detected in lymph nodes, spleen, thymus, intestine, liver and lung in the absence of clinical signs (Tischer et al., 1986; Allan et al., 1995; Fenaux et al., 2003). Despite the absence of signs and lesions, challenged animals were able to elicit specific antibodies to PCV-1 (Tischer et al., 1986; Allan et al., 1995; Tischer et al., 1995). Only one report has suggested that PCV-1 can replicate efficiently in lungs, causing hemorrhages in porcine fetuses inoculated at 55 days of gestation (Saha et al., 2011).

PCV-1 antibodies are found in pigs worldwide, from all production phases and to a variable frequency (Allan et al., 1994; Dulac and Afshar et al., 1989; Tischer et al., 1982; Muhlig et al., 2006). Although the virus is of ubiquitous nature, the higher prevalence of seropositive pigs during the post-weaning period suggested the nursery phase as the most probable period of infection (Tischer et al., 1995).

1.3 Porcine circovirus 2

1.3.1 History

In 1991, a new clinical-pathological condition in pigs named post-weaning multisystemic wasting syndrome (PMWS) and characterized by wasting and microscopic lymphoid lesions was detected in Canada (Clark, 1997; Harding, 1997). Subsequently, a distinct circovirus from the previously known as PCV (nowadays PCV-1) was found through electron microscopy, immunohistochemistry (IHC) and *in situ* hybridization (ISH) in the lesions of diseased animals (Ellis et al., 1998; Allan et al., 1998; Rosell et al., 1999). The genome of this new circovirus was far different from the non-pathogenic PCV-1 and the novel pathogen was designated as PCV-2 (Meehan et al., 1998). A few years later, the virus was found to be spread in many countries around the world, causing great economical losses to the pig production sector (Segalés et al., 2013). Although the syndrome caused by PCV-2 was firstly described in 1991, retrospective studies showed that the virus was already present in pig tissues since 1962 and in PMWS lesions since 1985 (Jacobsen et al. 2009).

Despite the systematic presence of the virus in diseased animals (Clark, 1997; Harding, 1996; Rosell et al., 1999), the perception about causality was not unanimous in the scientific and veterinary world for a quite long period (Segalés et al., 2013). Such discrepancies were probably due to the multifactorial nature of PMWS and the lack of consistent disease reproduction through the first experimental infections (Balasch et al.,

1999; Allan et al., 1999). The controversy on PCV-2 pathogenicity disappeared once vaccines against the virus came into the worldwide market and drastically reduced PMWS outbreaks preventing the associated economic losses (Segalés, 2015; Karuppannan and Opriessnig, 2017).

Besides PMWS, PCV-2 is involved in other swine clinical conditions collectively called porcine circoviruses diseases (PCVDs) (Segalés, 2012). PMWS is currently known as PCV-2 systemic disease (PCV-2-SD), while the others conditions are named as PCV-2 reproductive disease (PCV-2-RD), PCV-2 subclinical infection (PCV-2-SI), and porcine dermatitis and nephropathy syndrome (PDNS) (Segalés, 2012).

1.3.2 Genotypes

PCV-2 has a high mutation rate, close to the one of single stranded RNA viruses (Firth et al., 2009). The genetic variability of this virus led to different proposals of sub-classifications beyond the species level; four major genotypes of PCV-2 were initially defined (PCV-2a to PCV-2d) (Segalés et al., 2008). However, over time, some new genotypes have been suggested such as PCV-2e (Harmon et al., 2015; Davies et al., 2016) and PCV-2f (Bao et al., 2018). A new classification in eight genotypes was further proposed, from PCV-2a to PCV-2h (Franzo and Segalés, 2018), and a new genotype has been recently suggested, PCV-2i (Wang et al., 2020). Indeed, considering the PCV-2 genomic organization, efficient immune responses from pigs against the virus, vaccination pressure and recombination capabilities, the emergence of new genotypes is expected in the future (Franzo et al., 2016).

PCV-2a was the most prevalent genotype circulating during the 1990s and was mainly linked with PCV-2-SD sporadic occurrence. By the end of 1990s and early 2000s, a change in genotype prevalence towards PCV-2b ("genotype shift") was described coinciding with epidemic forms of PCV-2 SD in America, Asia and Europe (Carman et al., 2006; Dupont et al., 2008; Cortey et al., 2011). In contrast, PCV-2c was firstly described in a retrospective study in samples from 1980s from Denmark (Dupont et al., 2008) and, later on, occasionally described in Brazil (Franzo et al., 2015) and China (Liu et al., 2016). During last years, a second genotype shift from PCV-2b to PCV-2d has occurred globally (Xiao et al., 2016; Franzo and Segalés, 2018), although reasons behind such change in genotype prevalence are currently unknown. At field level, PCV-2 infection with more than one

genotype in the same farm as well as in the same pig has been described (Hesse et al., 2008; Grau-Roma et al., 2008).

1.3.3 Epidemiology

PCV-2 is a ubiquitous virus able to infect domestic pigs and other members of the *Suidae* family, such as feral pigs (Franzo et al., 2015), wild boars (Ellis et al., 2003) and peccaries (de Castro et al., 2014). Rodents (Kiupel et al., 2005; Csagola et al., 2008; Lorinez et al., 2010) and flies (*Musca domesticus*) (Blunt et al., 2011) have been described as potential vectors for the virus spread.

Considering the widespread nature of PCV-2 (Segalés et al, 2013), the probability of a non-vaccinated animal to be infected by PCV-2 in its lifetime is high. PCV-2 can be shed through several routes (Patterson and Opriessnig, 2010; Patterson et al., 2011), and it can be found in saliva, serum, urine, colostrum, milk, semen and nasal, bronchial and ocular secretions (Sibila et al., 2004; Segalés et al., 2005; Patterson and Opriessnig et al., 2010). Thus, viral infection may occur indirectly by infected living vectors, fomites and aerosols, but the most important transmission routes are horizontal (pig/sow-to-pig by nose contact) and vertical (intrauterine) ones (Segalés et al., 2005; Madson and Opriessnig, 2011).

1.3.4 Disease association

Despite the association of PCV-2 with the systemic disease was firstly characterized in 1991 in Canada (Clark, 1997; Harding, 1996), pathogenesis of PCVDs is not completely elucidated so far.

PCV-2 is primarily found in lymphoid tissues (Rosell et al., 1999) and, in consequence, the animals that develop PCV-2-SD develop characteristic lymphocyte depletion and granulomatous lesions in lymphoid organs (Allan et al., 1998; Rosell et al. 1999; Sánchez et al., 2003; Yu et al., 2007). PCV-2 SD is a common problem in the late nursery and early growing phases (Harding and Clark, 1997). PCV-2-SI is characterized by lack of evident clinical signs but decrease in the average daily weight gain (Young et al., 2011). The viral infection can occur at embryonic stage, but also at mid-late gestation, causing reproductive problems collectively named PCV-2-RD (Madson and Opriessnig, 2011; Segalés, 2012). PDNS can affect pigs at all stages of production, although mainly growing animals (Drolet et al., 1999). The clinical manifestation and main pathological findings found in PCVDs are displayed in Table 1.2.

Table 1.2. Most common clinical signs and pathological findings of PCVDs (Segalés, 2012).

PCVD	Clinical Signs	Macroscopic lesions	Microscopic lesions
PCV-2-SI	Low gain weight	No lesions	No lesions or minimal lymphocyte depletion and/or granulomatous inflammation of lymphoid tissues
PCV-2-SD	Wasting, jaundice, respiratory and/or digestive signs	Enlargement of lymph-nodes, interstitial nephritis, interstitial pneumonia and catarrhal enteritis	Moderate to severe lymphocyte depletion, granulomatous inflammation of lymphoid tissues; lymphohistiocytic infiltrates in lung, kidney, liver, and intestines
PCV-2-RD	Reproductive failures (abortion, mummified and stillborn piglets); probable return-to-estrus	Mummified fetuses, stillborn piglets, dead fetuses with cardiac hypertrophy, hydrothorax and/or hydropericardium	Fibrous and/or necrotizing myocarditis and pneumonia in fetuses
PDNS	Red papules and macules on skin	Cutaneous lesions (hemorrhagic and necrotic) and petechiae in kidney cortical area	Mild lymphocyte depletion and granulomatous inflammation of lymphoid tissues; necrotic and/or fibrinous glomerulonephritis; systemic necrotizing vasculitis

1.3.5 Diagnosis and control

The laboratory assessment of PCV-2 infection can be done through the detection of viral genome and/or antigen. However, due to the widespread distribution of the virus, the mere presence of the virus is not enough to diagnose the clinical conditions caused by PCV-2 (Segalés, 2012). Grau-Roma et al. (2012) proposed a herd case definition for PCV-2-SD, which was based on two major criteria: 1) significant increase of mortality in post-weaning pigs associated with clinical signs compatible with PCV-2-SD, mainly wasting and respiratory problems, and 2) confirmation of PCV-2-SD by means of individual diagnostic criteria in at least 1 out of 3-5 necropsied pigs. The individual diagnosis has in turn three main criteria: 1) presence of PCV-2-SD compatible clinical signs, 2) moderate to severe lymphocyte depletion with granulomatous inflammation of lymphoid tissues, and 3)

detection of moderate to high amount of PCV-2 within the lymphoid lesions (Segalés, 2012). Subsequently, specific diagnostic criteria were established for each PCVD (Segalés, 2012). The presence of clinical signs together with histopathological lesions associated to the presence of the agent are key elements for the diagnosis. Thus, the IHC and ISH are laboratory techniques widely used to detect the presence of the virus within the damaged in tissue (Rosell et al., 1999).

PCR and quantitative PCR (qPCR) are the most used techniques to detect the viral genome. The qPCR technique allows quantifying the amount of virus in different samples. This latter point is important, since it has been described that animals suffering from PCVD have higher amount of virus (around or higher than 10^7 copies/mL of serum, depending on the qPCR technique used) than subclinical infected ones (Grau-Roma et al., 2009; Segalés, 2012).

The ubiquitous nature of this virus prevents to use serological tests to diagnose PCVDs; however, enzyme-linked immunosorbent assays (ELISAs), immunoperoxidase monolayer assay (IPMA) and immunofluorescent assay (IFA) can be used for PCV-2 antibody surveillance to monitor evidence of natural infection, presence of maternally derived antibodies or seroconversion due to vaccination (Allan et al., 1999; Sun et al., 2010; Fraile et al., 2012; Feng et al., 2014).

The control of PCV-2 associated clinical manifestations, it is mainly done by herd vaccination (Segalés, 2015). Nowadays, several commercial vaccines against PCV-2 are available, and most of them are based on PCV-2a genotype; however, these vaccines have been shown efficient against the other circulating genotypes, namely PCV-2b and PCV-2d (Opriessnig et al., 2014). Current vaccines available in the market are based on 1) inactivated virus, 2) chimeric viruses (inactivated PCV-1 expressing the PCV-2a Cap protein), and 3) subunit (Cap protein expressed in baculovirus system). These vaccines can decrease the viral loads in the animals, thus reducing the clinical manifestation of the virus (Reynaud et al., 2004; Yang et al., 2012). In addition, PCV-2 vaccines have demonstrated to significantly decrease the economic impact caused by the PCV-2-SI by improving the average daily weight gain (Kurmann et al., 2011; Young et al., 2011). Some other measures, such as good management practices (Madec et al 2008), co-infection control (Opriessnig and Halbur, 2012) and breeding selection (Opriessnig et al., 2009), can also help diminishing the disease impact in the field. In fact, these measures were capital at the time when no PCV-2 vaccines were available.

1.4 Porcine circovirus 3

1.4.1 History

PCV-3 was discovered in 2015 in USA by next-generation sequencing (NGS) methods in 3- to 9-week-old pigs suffering from different clinical conditions (Phan et al., 2016; Palinski et al., 2017). Viral genome was found in animals affected by digestive disorders, respiratory and neurological signs, cardiac and multisystemic inflammation, reproductive failure, and a PDNS-like condition (Phan et al., 2016; Palinski et al., 2017).

Since its first description, PCV-3 has been detected in pigs from different countries and in animals displaying a number of different clinical-pathological outcomes, such as respiratory disease (Zhai et al., 2017; Kedkovid, et al., 2018b; Shen et al., 2018; Qi et al., 2019), digestive disorders (Zhai et al., 2017; Qi et al., 2019), congenital tremors (Shen et al., 2018), rectal prolapse (Phan et al., 2016), reproductive problems (Faccini et al., 2017; Arruda et al., 2019; Deim et al., 2019), and multisystemic inflammation (Phan et al., 2016; Arruda et al., 2019). Additionally, this novel virus has been detected in healthy animals of different ages and countries (Stadejek et al., 2017; Zhai et al., 2017; Klaumann et al., 2018a; Klaumann et al., 2018b; Klaumann et al., 2019; Franzo et al., 2018; Ye et al., 2018; Saporiti et al., 2020).

Although the virus was firstly described in 2015, retrospective studies found that PCV-3 was already circulating since 1967 in Brazil (Rodrigues et al., 2020), 1993 in Sweden (Ye et al., 2018) and since 1996 in Spain and in China (Klaumann et al., 2018a; Sun et al., 2018). It is thought that the virus may have emerged around the 1960s (Fu et al., 2018; Saraiva et al., 2019) because of a recombination of other circoviruses (Ku et al., 2017; Franzo et al., 2018b).

1.4.2 Genotypes

To date, there are 644 complete genome and 1030 capsid gene sequences of PCV-3 available on GenBank (June 3rd, 2021. Available in https://www.ncbi.nlm.nih.gov/nuccore). The similarity among PCV-3 available sequences is high, ranging from 97% to 100% for the complete genome and from 94% to 100% for the Cap gene. This high similarity makes difficult to establish further sub-classification from species to genotypes (Zheng et al., 2017; Klaumann et al., 2018b; Fux et al., 2018; Zou et al., 2018; Franzo et al., 2020). While for PCV-2 it was verified that the *Cap* gene sequencing is suitable for genotype classification (Olvera et al., 2007), different classifications based on potential marker positions located in

the amino acids (aa) sites from ORF2 only or ORF1 and ORF2 have been proposed for PCV-3 (Fu et al., 2018; Fux et al., 2018; Li et al., 2018; Franzo et al., 2020). Fux et al. (2018), by analyzing complete genome and ORF2 gene sequences available at GenBank, proposed two main groups (PCV-3a and PCV-3b) where the aa sites S122A (ORF1), and A24V and R27K (ORF2) determined the clade divisions. The same authors also proposed the sub-groups *a1, a2, b1* and *b2*; however, the sub-group *a2* had aa motifs shared between groups *a* and *b* (Fux et al., 2018). Li et al. (2018) analyzed the complete coding sequences (ORF1 + ORF2) and also proposed two main groups (PCV-3a and PCV-3b) based on the same already mentioned codons. In this study, group *a* was subdivided in *a1* and *a2*, but some strains were randomly distributed and were classified as intermediate (IM). It was also proposed a possible different antigenicity between groups *a* and *b* due the difference of aa site 24 (ORF2) in each group. Due to the lack of consistency when analyzing only ORF2, these authors recommended analyzing the complete coding sequences (Li et al., 2018). On the contrary, Fu et al. (2018) analyzed phylogenetically only the ORF2 gene and proposed three main groups (PCV-3a, PCV-3b and PCV-3c) based on two aa mutations of the Cap gene (A24V and R27K). In this classification, strains with alanine (A) at position 24 and arginine (R) at position 27 would belong to PCV-3a, while strains with arginine (A) and lysine (K) would belong to PCV-3b, and a putative PCV-3c would have valine (V) and lysine (K) at positions 24 and 27 (Fu et al., 2018). The authors further subdivided the group *a* in *a1*, *a2* and *a3* based on evolutionary analyses and/or other aa mutations (Fu et al., 2018).

Despite the previous classification proposals, a very recent study highlighted several exceptions for the mentioned marker positions and suggested a definition based on a single PCV-3 genotype, the PCV-3a (Franzo et al., 2020). The new proposal suggests that sequences with aa maximum genetic distance of 3% and 6% at complete genome and Cap gene levels, respectively, and bootstrap support higher than 90% would belong to the same genotype (Franzo et al., 2020). This proposal intended to be a consensus among different worldwide experts on PCV-3, and although two sequences from China may fall into the category of a putative PCV-3b genotype, the very limited number of them and the impossibility to re-check the sequences prevented the definitive establishment of such second genotype to date.

Finally, up to now there are no evidence supporting a relationship between PCV-3 variability and any specific disease or virus pathogenicity (Arruda et al., 2019; Mora-Díaz et al., 2020)

1.4.3 Epidemiology

Shortly after the initial detection of the PCV-3 genome in USA (Phan et al., 2016; Palinski et al., 2017), viral nucleic acid was demonstrated in domestic pigs samples from different countries worldwide (Faccini et al., 2017; Stadejek et al., 2017; Franzo et al., 2018a; Hayashi et al., 2018; Tochetto et al., 2018; Saporiti et al., 2020). Indeed, the virus was also found in moderate to high prevalence in wild boar (*Sus scrofa scrofa*) without any association with clinical conditions (Klaumann et al., 2018c; Franzo et al., 2018c; Prinz et al., 2019; Tan et al., 2020). Indeed, a recent study demonstrated evidence of a long-term infection in this species, which may suggest wild boar as a potential reservoir for the virus (Klaumann et al., 2018c).

PCV-3 genome has been detected in domestic pigs from all production phases such as in dams, suckling piglets, weaners, and growing-finishing pigs, also including mummified and stillborn piglets (Palinski et al., 2017; Arruda et al., 2019; Klaumann et al., 2019; Woźniak et al., 2020). The distribution of PCV-3 positivity in the different production phases as well as the frequency of detection can be observed in Tables from 1.3 to 1.8. The duration of viremia has been poorly studied. A longitudinally study from four Spanish farms sampling pigs from 3-4 weeks of age to slaughter showed detection of PCV-3 genome in a number of animals, and few pigs had an apparent long-term infection (4-23 weeks of duration) (Klaumann et al., 2019). Noteworthy, frequency of viral nucleic acid detection did not show association to any specific age (Klaumann et al., 2019).

From its first description, viral DNA has also been detected in non-Suidae species such as dogs, mice, cattle, chamois, roe deer, red deer, fallow deer, mouflon, ticks and mosquitoes (Franzo et al., 2019; Jiang et al., 2019; Sun et al., 2019; Wang et al., 2019; Czyżewska-Dors et al., 2020; Ha et al., 2020). Although the very sporadic presence of PCV-3 DNA in these species, there is no evidence of viral pathogenicity in them. However, the presence of the virus may suggest a potential spillover between species, a subject that must be further investigated.

The detection of PCV-3 in four baboons has been associated to a xenotransplantation experiment using pig hearts subsequently confirmed to be PCV-3 positive by PCR (Krüger et al., 2019). After 182 and 195 days of survival time of transplant, high amount of viral genome was found in organs such as spleen, liver, lung, kidney, skin, and muscle, in two out of the four transplanted baboons, suggesting viral replication. The other two transplanted baboons, which had shorter survival time after transplant (15 and 27 days), were found PCR

negative to PCV-3 in the same analyzed organs, apart from the transplanted pericardium, which was still PCV-3 PCR positive. However, the isolation of PCV-3 attempted in human cells in the same study was unsuccessful (Krüger et al., 2019).

The PCV-3 pattern of antibody dynamics is, up to now, unknown. Serological surveillance is essential to obtain information on the immune response generated against PCV-3 infection. However serological tests (ELISA) are available in few laboratories and they are used just for research purposes (Palinski et al., 2017; Deng et al., 2018; Mora-Diaz et al., 2020). A Chinese field study, showed that the percentage of seropositive pigs increased from 22.3% (76/370) to 51.9% (152/293) in samples taken in 2015 and 2017, respectively (Deng et al., 2018). Another small survey in USA found a 56.6% (47/83) of PCV-3 seropositive samples sent for other diagnostic purposes from multiple states (Palinski et al., 2017).

In conclusion, PCV-3 evidence of infection is present worldwide at different ages and frequency of detection. However, more studies are needed to elucidate the pattern of PCV-3 circulation and seroconversion in swine farms.

1.4.4 Disease association

Since the first PCV-3 description, the viral DNA has been identified in samples from pigs with several different disease conditions. Noteworthy, detection of PCV-3 genome in a sick animal does not preclude that this virus is the cause of the clinical signs or lesions. Moreover, the lack of proper negative controls (age-matched, healthy pigs) to compare with in most of these studies further complicates the interpretation of the viral infection regarding the causality of the disease problem.

Literature linking PCV-3 with different disorders is extensive already, mainly regarding respiratory, digestive, reproductive and neurological disorders. However, most of these studies do not provide information on viral genome detection in healthy pigs from the same farms.

1.4.4.1 Respiratory disease

Several reports have been published on PCV-3 infected pigs displaying respiratory clinical signs (Table 1.3). In some of them, histologic lesions were reported while in others only clinical signs or eventually gross lesions were described.

1.4.4.2 Enteric disease

So far, two studies have detected PCV-3 DNA in samples from pigs presenting digestive disorders (Table 1.4). In both studies, no pathologic investigations were performed.

1.4.4.3 Reproductive disease

PCV-3 has also been found in farms with pigs displaying reproductive problems, as listed in Table 1.5. In many of these studies, no histological investigations were performed; moreover, detailed clinical history of analyzed farms was not given.

Despite the important number of studies reporting the presence of the virus in farms presenting an increase of aborted fetuses, stillborn and mummified piglets, the detection rate of PCV-3 in farms with standard number of stillborn and mummified piglets has not been thoroughly investigated. Therefore, the potential background infection with PCV-3 in normally performing farms is still unknown.

1.4.4.4 Neurological disease

PCV-3 has been found in pigs suffering from neurological signs or congenital tremors (Table 1.6). Histology was performed in three out of the four studies. Noteworthy, *Atypical porcine pestivirus* (APPV) was also found in 5 out of 7 of the animals tested by Chen et al. (2017) and in 3 out of 3 examined by Williamson et al. (2021).

1.4.4.5. Other conditions

PCV-3 has also been presumably associated to other disease conditions (Table 1.7) than the ones previously mentioned in tables 1.3 to 1.6.

1.4.4.6. Healthy animals

As indicated previously, a significant number of studies did not test healthy animals as a control group. Such approach makes difficult to reach consistent conclusions on the causality between PCV-3 infection and disease, especially when no lesions are characterized, and the presence of virus cannot be unequivocally linked to observed lesions. Indeed, the virus has also been detected in samples from healthy animals all around the world (Table 1.8).

1.4.5 Co-Infections

Being a ubiquitous virus, PCV-3 has been found in co-infection with other pathogens by many studies (Table 1.9). The existence of such co-infections in diseased animals, emphasizes the need to study the eventual pathogenicity of PCV-3 in presence and absence of other infectious agents. At least when mirroring with PCV-2, co-infection of this virus with other pathogens leads to more severe disease presentation under field (Opriessnig and Halbur, 2012) and experimental (Tomás et al., 2008) conditions.

Table 1.3. List of respiratory problems reported in animals positive by PCV-3 PCR in at least one of the tested samples.

Clinical Signs / Lesions	Production phase	Tested samples	% and Proportion of PCV-3 PCR positivity in:		Country	Reference
			Diseased animals	Healthy animals		
Respiratory disease with dyspnea / diffuse moderate lymphohistiocytic interstitial pneumonia and acute bronchitis	Lactating / nursery / fattening	Tissues*	100.0% (3/3) **	NI	USA	Phan et al., 2016
Respiratory disease	NA	Lung homogenate / oral fluid / nasal swab	12.5% (34/271)	NI	USA	Palinski et al., 2017
	Lactating	Lung tissues	26.6% (25/94)	0.0% (0/42) ***	China	Qi et al., 2019
Severe respiratory disease	Nursery	Sera	63.7% (51/80)	1.85% (4/216)	China	Zhai et al., 2017
Mild respiratory disease	Nursery	Sera	13.1% (23/175)	1.85% (4/216)	China	
Abdominal breathing / lung swelling and congestion	Nursery	Tissues / sera	NA****	NI	China	Shen et al., 2018
Porcine respiratory disease complex related signs	Growing	Sera	60.0% (15/25)	28.0% (7/25)	Thailand	Kedkovid et al., 2018b
Porcine respiratory disease complex / broncho-interstitial pneumonia	Growing	Lung and lymph node tissues	62.5% (5/8)	NI	Thailand	
Respiratory distress / bronchointerstitial pneumonia and infiltrating lymphocytes	Growing	Tissues	100% (2/2)	NI	South Korea	Kim et al., 2018a

NI: Not included in the published manuscript; NA: Non-available information in the published manuscript.

*Not specified; **NGS results; ***The type of samples analyzed in control animals (feces) was different from the ones used in diseased pigs (lung tissues); ****Number of tested samples not included in the published manuscript.

Table 1.4. Digestive disorders reported in PCV-3 PCR positive animals.

Clinical Signs / Lesions	Production phase	Tested samples	% and Proportion of PCV-3 PCR positivity in:		Country	Reference
			Diseased animals	Healthy animals		
Diarrhea	Nursery	Fecal samples	17.14% (6/35)	2.86% (1/35)	China	Zhai et al., 2017
Diarrhea / vomiting	Lactating	Intestinal tissues / fecal samples	10.4% (50/480)	0.0% (0/42) *	China	Qi et al., 2019

*The type of samples analyzed in control animals (feces) was different from the ones used in diseased pigs (intestinal tissues).

Table 1.5. Reproductive problems reported in PCV-3 PCR positive animals.

Clinical Signs / Lesions	Production phase	Tested samples	% and Proportion of PCV-3 PCR positivity in:		Country	Reference
			Diseased animals	Healthy animals		
Reproductive failure	Gestation	Sera from sows	45.9% (39/85)	21.9% (23/105)	China	Zou et al., 2018
		Pool of tissues from aborted fetuses / Pool of tissues from stillborn piglets	100.0% (2/2)	100.0% (2/2)	Italy	Faccini et al., 2017
		Tissues from mummified fetuses	97.0% (270/276)	NI	Brazil	Dal Santo et al., 2020
Sow mortality and reproductive failure (aborted mummifies fetuses)		Sow tissues / fetal tissues	NA	NI	USA	Palinski et al., 2017
Sows delivering stillbirth piglets		Pool of sera from sows	100.0% (2/2)	0.0% (0/2)	Brazil	Tochetto et al., 2018
		Sera sows	67.4% (31/46)	60.5% (26/43)	Brazil	
Acute losses in neonatal piglets / increase of rate of stillborn / sow mortality		Stillborn / tissues / semen / sera	34.7% (77/222)	NI	China	Ku et al., 2017

Table 1.5. (Continuation) - Reproductive problems reported in PCV-3 PCR positive animals.

Abortion / death of suckling piglets	Gestation / Lactating	Tissues from aborted fetuses / weak suckling piglets	36.4% (8/22)	NI	South Korea	Kim et al., 2018a
Acute loss of neonatal piglets		Tissues from aborted fetuses / stillborn / weak-born piglets	89.0% (49/55)	NI	Hungary	Deim et al., 2019
Reproductive failure / weak-born neonatal piglets / myocarditis / encephalitis		Tissues from fetuses / suckling	100.0% (25/25)	NI	USA	Arruda et a., 2019

NA: Non-available information in the published manuscript, NI: Not included in the published manuscript.

Table 1.6. Neurological signs reported in PCV-3 PCR positive animals.

Clinical Signs / Lesions	Production phase	Tested samples	% and Proportion of PCV-3 PCR positivity in:		Country	Reference
			Diseased animals	Healthy animals		
Neurological signs	Lactating	Tissues	100.0% (1/1)	NI	USA	Phan et al., 2016
Congenital tremors	Lactating	Tissues	100.0% (7/7)	NI	China	Chen et al., 2017
Tremors, weak-born neonatal piglets / myocarditis, encephalitis, gliosis and lymphocytic perivascular cuffing	Lactating	Tissues	100.0% (2/2)	NI	USA	Arruda et a., 2019
Tremors, neurological signs in piglets after birth and multisystemic inflammation / non-suppurative encephalomyelitis	Lactating	Tissues	100.0% (3/3)	NI	UK	Williamson et al., 2021

NI: Not included in the published manuscript.

Table 1.7. Other conditions presumable associated to reportedCV-3 infection.

Clinical Signs / Lesions	Production phase	Tested samples	% and Proportion of PCV-3 PCR positivity in:		Country	Reference
			Diseased animals	Healthy animals		
Myocarditis / periarteritis	Lactating / nursery / fattening	Tissues	100.0% (3/3)*	NI	USA	Phan et al., 2016
PDNS	NA	Tissues	93.8% (45/48)	NI	USA	Palinski et al., 2017
	Sows	Pooled tissues	NA*	NI	USA	Palinski et al., 2017
PDNS / acute deaths / myocarditis / arteritis / periarteritis	Nursery	Tissues	100.0% (11/11)	NI	USA	Arruda et al., 2019
Arthrogryposis	Stillborn piglets	Tissues	100.0% (4/4)	NI	UK	Williamson et al., 2021

*NGS results; NA: Non-available information in the published manuscript, NI: Not included in the published manuscript.

Table 1.8. Healthy animals reported as PCV-3 PCR positive.

Clinical Signs / Lesions	Production phase	Tested samples	% and Proportion of PCV-3 PCR positivity in healthy animals	Country	Reference
Asymptomatic	Weaning / growing / finishing	Oral fluids	43.4% (142/327)	South Korea	Kwon et al., 2017
Asymptomatic	Sows / fetuses	Tissues	59.5% (132/222)	China	Zheng et al., 2017
Asymptomatic	Sows (in lactation)	Sera	47.3% (18/38)	Thailand	Kedkovid et al., 2018a
Asymptomatic	Different production phases	Tissues and sera	56.4% (44/78)	Denmark	Franzo et al., 2018a
Asymptomatic	Different production phases	Tissues, sera and nasal swabs	37.4% (37/99)	Italy	
Asymptomatic	Different production phases	Pool of sera	15.0% (14/94)	Spain	
Asymptomatic	NA	Lymph node tissues	NA	Sweden	Ye et al., 2018
Asymptomatic	Growing	Tissues, serum and nasal swabs	5.9% (5/90)	Poland	Stadejek et al. 2017
Asymptomatic	Nursery / Finishing	Sera	10% (7/73)	Spain	Klaumann et al., 2018a
Asymptomatic	Nursery / Finishing	Sera	6.4% (7/110)	Spain	Saporiti et al., 2020
Asymptomatic	Nursery / Finishing	Sera	13.0% (13/100)	Belgium	
Asymptomatic	Nursery / Finishing	Sera	10.4% (7/67)	France	
Asymptomatic	Nursery / Finishing	Sera	6.3% (5/80)	Germany	
Asymptomatic	Nursery / Finishing	Sera	4.5% (3/67)	Italy	
Asymptomatic	Nursery / Finishing	Sera	6.3% (5/80)	Denmark	
Asymptomatic	Nursery / Finishing	Sera	14.0% (7/50)	The Netherlands	
Asymptomatic	Nursery / Finishing	Sera	4.0% (2/50)	Ireland	
Asymptomatic	Nursery / Finishing	Sera	15.0% (3/20)	Sweden	

NA: Non-available information in the published manuscript.

Table 1.9. List of pathogens found concomitantly with the presence of PCV-3.

Pathogen	**Country**	**% and Proportion of of co-infection**	**Reference**
PCV-2	China	15.8% (35/222)	Ku et al.,2017
		39.4% (52/132)	Zheng et al., 2017
		30.0% (3/10)	Sun et al., 2018
		70.0% (28/40)	Zhao et al., 2018
		1.9% (3/159)	Chen et al., 2019
		6.78% (32/472)	Xia et al., 2019
	South Korea	28.3% (13/46)	Kim et al., 2017
		19.3% (11/57)	Kim et al., 2018b
	Thailand	20.0% (1/5)	Kedkovid et al., 2018b
	Poland	4.8% (8/166)	Wozniak et al., 2019
	USA	5.4% (115/2125)	Wang et al., 2019)
	Spain	0.9% (1/110)	Saporiti et al., 2020
	Belgium	4.0% (4/100)	
	France	10.4% (7/67)	
	Italy	1.5% (1/67)	
	Denmark	2.5% (2/80)	
	Ireland	2.0% (1/50)	
	Brazil	78.3% (216/276)	Dal Santo et al., 2020
	Colombia	24.0% (12/50)	Vargas-Bermúdez et al., 2021
Porcine reproductive and respiratory syndrome virus (PRRSV)	Thailand	20.0% (1/5)	Kedkovid et al., 2018b
	South Korea	100.0% (2/2)	Kim et al., 2018a
		43.8% (25/57)	Kim et al., 2018b
	China	0.6% (1/159)	Chen et al., 2019

Table 1.9. (Continuation) - List of pathogens found concomitantly with the presence of PCV-3.

Porcine parvovirus (PPV)	China	20.0% (8/40)	Zhao et al., 2018
	Brazil	58.7% (162/276)	Dal Santo et al., 2020
Classical swine fever virus (CSFV)	China	90.0% (9/10)	Sun et al., 2018
		2.5% (1/40)	Zhao et al., 2018
Porcine epidemic diarrhea virus (PEDV)	China	NA	Chen et al., 2017
Atypical porcine pestivirus (APPV)	China	NA	
	UK	42.8.% (3/7)	Williamson et al., 2021
Porcine kobuvirus (PKV)	China	NA	Chen et al., 2017
Porcine pseudorabies virus (PRV)	China	NA	
		5.0% (2/40)	Zhao et al., 2018
Porcine sapelovirus (PSV)	China	NA	Chen et al., 2017
P*orcine bocavirus* (PBoV)	China	NA	
Torque teno sus virus (TTSuV1 and 2)	China	50% (66/132)	Zheng et al., 2018
Streptococcus spp	USA	NA	Phan et al., 2017
	Thailand	20.0% (1/5)	Kedkovid et al., 2018b
	South Korea	100.0% (2/2)	Kim et al., 2018a
Glaeserella parasuis	USA	NA	Phan et al., 2017
Mycoplasma hyorhinis	USA	NA	
Mycoplasma hyopneumoniae	South Korea	100.0% (2/2)	Kim et al., 2018a
Pasteurella multocida	Thailand	NA	Kedkovid et al., 2018b
Leptospira spp	Brazil	9.4% (26/276)	Dal Santo et al., 2020

NA: Non-available information in the published manuscript.

1.4.6 Pathogenesis

The pathogenicity of PCV-3 is not well established so far. As mentioned before, the viral detection in animals suffering from certain clinical conditions is not sufficient to demonstrate the involvement of this agent in such problems. One of the major limitations to study PCV-3 pathogenesis has been the lack of an isolate to attempt experimental infections. Early studies tried to isolate the virus from damaged PCV-3 PCR positive tissues with unsuccessful results (Faccini et al., 2017; Palinski et al., 2017). However, two research groups were finally able to isolate PCV-3 in cell culture in 2020. The first research group isolated the virus in primary PK cells using a lymph node containing a high amount of PCV-3 DNA from a clinically healthy pig as inoculum (Oh and Chae, 2020). The virus did not produce cytopathic effect, but the presence of the agent was confirmed by ISH (Oh and Chae, 2020). Shortly after, another research group was able to isolate PCV-3 in PK-15 cells using tissues from fetuses and newborn piglets from farms with reproductive losses as inoculum (Mora-Díaz et al., 2020). This latter research group also performed an experimental infection with the isolated virus (Mora-Díaz et al., 2020) as described in Table 1.10. In this latter table, the other two experimental infections published so far using tissue homogenate (Temeeyasen et al., 2021) or an infectious clone (Jiang et al., 2019) as inoculum are also described.

Only in one out of these three experimental studies, the inoculation of PCV-3 resulted in clinical signs (Jiang et al., 2019). However, in all three studies the animals showed histopathologic lesions at necropsy at the end of the experiment (Jiang et al., 2019; Mora-Díaz et al., 2020; Temeeyasen et al., 2021), although in a wide range of frequency. While all inoculated animals had histopathologic lesions in the study performed by Jiang et al. (2019), in the other two studies, only 50% of the inoculated pigs showed histopathological lesions (Mora-Díaz et al., 2020; Temeeyasen et al., 2021). Therefore, the knowledge acquired on PCV-3 pathogenicity and pathogenesis with these few experimental studies is very limited. Moreover, these experimental infections provided some information, somehow contradictory, with regards viremia and the immune response against PCV-3 infection. In the study performed by Mora-Díaz et al. (2020) where pigs were inoculated with a PCV-3 isolate, all inoculated animals displayed viremia at 28 dpi and IgM antibodies were detected from days 7-14 until the necropsy day (28 dpi).

Table 1.10. PCV-3 experimental infections so far published, with the most significant results.

Reference	Inoculum	Volume, route of administration and dose	Animals			Clinical signs	Lesions	Tissues with positive PCV-3 ISH	Viremia	% and proportion of death animals
			Type	n	Age					
Jiang et al., 2019	Infectious PCV-3 DNA clone	2 mL IN of $10^{6.53}$ $TCID_{50}$/mL	SPF piglets	5	4 wo	Respiratory, digestive, anorexia, lethargy, PDNS - like skin lesions, shivering, and/or hyperspasmia	Moderate to severe gross and microscopic lesions of multisystemic inflammation	Lung, liver, ln, spleen, kidney, heart and SI	7 dpi up to 21 dpi	40% (2/5)
	Infectious PCV-3 DNA clone + KLH	2 mL IN of $10^{6.53}$ $TCID_{50}$/mL + 2 mL IN of $10^{6.53}$ $TCID_{50}$/mL after 4 days	SPF piglets	5	4 wo			Lung, liver, ln, spleen, kidney, heart and SI	7 dpi up to 21 dpi	40% (2/5)
	Control group	Sham inoculated	SPF piglets	5	4 wo	None	None	Negative	Negative	None
	Infectious PCV-3 DNA clone	2 mL IN of $10^{6.53}$ $TCID_{50}$/mL	SPF piglets	5	8 wo	PDNS-like disease	Moderate to severe gross lesions Mild to moderate microscopic lesions of multisystemic inflammation	Lung, liver, ln, spleen, kidney, heart and SI	7 dpi up to 21 dpi	None
	Control group	Sham inoculated	SPF piglets	5	8 wo	None	None	Negative	Negative	None

Table 1.10. (Continuation) - PCV-3 experimental infections so far published, with the most significant results.

Mora-Díaz et al., 2020	PCV-3 isolated in PK-15 cells from reproductive cases + KLH/IFA	1 mL IN and 1 mL IM of 6.6×10^{10} genomic copies/mL	CD/CD piglets	8	6 wo	No clinical signs	No gross lesions Mild multisystemic inflammation	Heart, kidney, intestine and arteries of the cited tissues	28 dpi	None
	Control group + KLH/IFA	Sham inoculated	CD/CD piglets	6	6 wo	No clinical signs	None	NA	Negative	None
Temeeyasen et al., 2021	Tissue homogenate PCV-3 qPCR and NGS positive	2 mL IN of 3.38×10^{12} ml^{-1} and 2 mL IM of 1.04×10^{11} ml^{-1} (Re-inoculation after 7 days)	CD/CD piglets	6	5 wo	No clinical signs	No gross lesions at 11, 21 or 42 dpi Mild multisystemic inflammation at 42 dpi	Heart, liver, spleen, kidney and SI	3 dpi up to 42 dpi	16.6% (1/6)*
	Tissue homogenate PCV-3 qPCR and NGS positive + KLH	2 mL IN of 3.38×10^{12} ml^{-1} and 2 mL IM of 1.04×10^{11} ml^{-1} (Re-inoculation after 7 days)	CD/CD piglets	6	5 wo	No clinical signs	No gross lesions at 11, 21 or 42 dpi Mild multisystemic inflammation at 42 dpi	Heart, liver, spleen, kidney and SI	3 dpi up to 42 dpi	None
	Control group	Sham inoculated	CD/CD piglets	3	5 wo	No clinical signs	None	Negative	Negative	None
	Control group + KLH/IFA	Sham inoculated + KLH	CD/CD piglets	3	5 wo	No clinical signs	None	Negative	Negative	None

IN: Intranasal; IM: Intramuscular; wo: weeks-old; Ln: Lymph node; SI: small intestine; KLH: keyhole limpet hemocyanin (immunostimulation); KLH/IFA: keyhole limpet hemocyanin emulsified in incomplete Freund's adjuvant (immunostimulation); SPF: specific pathogen free; CD/CD: caesarean-derived, colostrum-deprived; NA: non-available information in the published manuscript. *: One animal died during blood sampling at 11 dpi so necropsy was performed, thus in order to also verify gross and microscopic lesions at the same day in the other inoculated group one animal was randomly euthanized in order to be necropsied.

However, the animals did not seroconvert for IgG until 28 dpi. On the other hand, the pigs inoculated with a tissue homogenate with and without immunostimulant (Temeeyasen et al., 2021), had prolonged viremia (from 3 dpi until 42 dpi [the necropsy day]), did not present specific IgM antibodies but had detectable IgG (Temeeyasen et al., 2021).

Under field conditions, the strongest evidence of PCV-3 disease causality is its detection by ISH or IHC (and with the confirmed absence of other pathogens) in damaged tissues within lesions (Table 1.11). To date, ISH has been the most widely used technique on formalin-fixed, paraffin-embedded (FFPE) tissues; the very limited availability of antibodies (Palinski et al., 2017; Li et al., 2018) has prevented the development and validation of IHC techniques. As for example, whereas Phan et al. (2016) used the ISH to label PCV-3 positive cells in histological lesions such as myocarditis, Palinski et al. (2017) used the IHC to demonstrate the viral protein within PDNS-like lesions in skin, kidney and lymphoid tissues. Additionally, a recent report has found a strong association between the PCV-3 detection by ISH in lesions (myocarditis, PDNS, arteritis and/or encephalitis) of fetuses, weak-born and weaned piglets in several cases from USA (Arruda et al., 2019). In all the 36 cases, PCV-2 and PPV were absent and, PRRSV and APPV were only present in sporadic samples (3 and 2 cases, respectively). Unfortunately, despite the strong evidence of PCV-3 as the cause of lesions, virus isolation was not attempted in this study (Arruda et al., 2019). Moreover, in one study from South Korea, PCV-3 genome was detected by ISH in lung tissue from a pig suffering of respiratory distress and wasting (Kim et al., 2018a). However, this animal was also positive to other pathogens such as PRRSV, *Streptococcus suis* and *M. hyopneumoniae* (Kim et al., 2018a), compromising the idea of PCV-3 causality regarding the observed lesions. Similarly, in Thailand, a pig showing lung lesions resembling proliferative and necrotizing pneumonia was positive to PCV-3 by ISH. However, the animal was also positive to PRRSV, PCV-2, alpha-hemolytic *streptococcus* and *Pasteurella multocida* (Kedkovid et al., 2018b). Recently, in a Colombian farm experiencing reproductive disorders, one mummified and two weak born piglets delivered by a gilt contained high amount of PCV-3 DNA tested by ISH in lung and lymph node tissues (Vargas-Bermúdez et al., 2021

Table 1.11. Studies where PCV-3 genome or antigen has been detected in tissues within lesions from animals naturally infected in the field.

Clinical Signs	Lesions	Tissues	Stained cellular type	Technique	Country	Reference
Anorexia and weight loss	Myocarditis	Heart	Macrophages, sarcoplasm of myocytes, smooth muscles of arteries	ISH	USA	Phan et al., 2016
PDNS and reproductive failure	Necrotizing vasculitis with fibrinoid changes and lymphocytic infiltration, multifocal granulomatous lymphadenitis, glomerulonephritis	Skin, lymph node, kidney	Dermal intracytoplasmic lymphocytes, follicular and perifollicular intracytoplasmic lymphocytes, renal tubular epithelium	IHC	USA	Palinski et al., 2017
Respiratory distress and wasting	Perivascular and peribronchiolar lymphocytic infiltration	Lung	Alveolar macrophages	ISH	South Korea	Kim et al., 2018a
Porcine respiratory disease complex (PRDC)	Lesions resembling proliferative and necrotizing pneumonia	Lung	Lymphocytes	ISH	Thailand	Kedkovid et al., 2018b

Table 1.11. (Continuation) - Studies where PCV-3 genome or antigen has been detected in tissues within lesions from animals naturally infected in the field.

Reproductive failure / weak-born neonatal piglets	Myocarditis, encephalitis, perivasculitis, gliosis and lymphocytic perivascular cuffing	Placenta, heart, kidney, cerebrum	Trophoblasts, cardiac myocytes, smooth muscles of arteries, renal tubular epithelium, white and grey matter of the cerebrum, ependymal cells and neurons	ISH	USA	Arruda et al., 2019
PDNS / acute deaths	Epidermal necrosis, vasculitis, periarteritis and arteritis	Skin, heart, kidney, mesentery	Epidermis, follicular epithelium and arterioles in PDNS cases, myocytes, smooth muscles of arteries, renal tubular epithelium, adipocytes			
Reproductive failure	Necrotic changes, congestion, and inflammatory infiltrates in placenta, lymphoid depletion and granuloma, thickening of alveolar septa, interstitial nephritis	Placenta, lymph node, lung, kidney	Lymphoid follicles, macrophages-like cells in lung	ISH	Colombia	Vargas-Bermúdez et al., 2021

ISH: In situ hybridization, IHC: Immunohistochemistry.

All these naturally and experimentally PCV-3 infection studies have evidenced that the most common histological lesions associated to PCV-3 infection are multisystemic inflammation and myocarditis (Phan et al., 2016; Kedkovid et al., 2018b; Kim et al., 2018a; Arruda et al., 2019; Mora-Díaz et al., 2020), although lesions of PDNS have also been described (Palinski et al., 2017; Jiang et al., 2018; Arruda et al., 2019). In addition, the detection by ISH of the virus has provided information on the potential cell types supporting viral replication, being mainly myocardiocytes as well as the macrophage-like and vascular cells.

Besides the detection of PCV-3 nucleic acid or antigen within lesions, molecular biology techniques have been widely used to detect PCV-3 genome in different samples/tissues from *suidae* species (Table 1.12). Specifically, viral DNA has been detected in serum (Klaumann et al., 2018a; Kedkovid et al., 2018a; Tochetto et al., 2020), oral fluids (Franzo et al., 2018a), feces (Collins et al., 2017), semen (Ku e al., 2017), colostrum (Kedkovid et al., 2018a) and a number of different tissue samples, such as thymus, tonsil, lymph nodes, spleen, lung, kidney, liver, intestine, brain, placenta, uterus and fetal material (Palinski et al., 2017; Klaumann et al., 2018b; Wang et al., 2019; Woźniak et al., 2020).

Based on the studies listed in Table 1.12, it seems that the amount of PCV-3 DNA detected may vary depending on the sample type, study, the technique used as well as the clinical status of the pigs from which the samples were retrieved. In fact, the results reported by Arruda et al. (2019) indicate a potential correlation between clinical signs, gross and microscopic lesions with high viral loads (by qPCR and ISH). Indeed, in this study, reproductive cases harbored 10^5 to 10^{12} genomic copies/mL in thoracic tissues, perinatal mortality cases 10^{11} to 10^{12} genomic copies/mL of brain and finally, an average of 10^8 genomic copies/mL of lung tissues in weaned animals suffering from dermatopathy or sudden death.

Table 1.12. Comparison of viral loads in different sample types and different clinical conditions.

Samples	**Clinical signs**	**Viral loads of PCV-3 detection**	**Reference**
Sera	NA	From 10^2 to 10^4 copies/mL	Palinski et al., 2017
Tissues from sows	Sows with PDNS-like lesions and their respective fetuses	Approx. 10^4 copies/mL	
Tissues from fetuses		From 10^6 to 10^8 copies/mL	
Pool of lungs	Stillborn piglets from reproductive losses cases	10^{10} copies /mL	Faccini et al., 2017
Pool of tissues		10^9 copies /mL	
Serum from sows	Healthy sows	Approx. 10^5 copies/mL	Kedkovid et al., 2018a
Colostrum from sows		Approx.. 10^5 copies/mL	
Serum	Porcine respiratory disease complex affected pigs	Approx. 10^3 copies/mL	Kedkovid et al., 2018b
Serum	Healthy pigs	Approx. 10^1 copies/mL	
Serum	Retrospective study from Spanish pigs (healthy and diseased)	From 10^5 to 10^{10}copies/mL	Klaumann et al., 2018a
Serum	Healthy animals from 4-23 weeks old	BQL ($<10^3$ copies/mL)	Klaumann et al., 2019

Table 1.12. (Continuation) - Comparison of viral loads in different sample types and different clinical conditions.

Thoracic tissues	Reproductive failure cases	From 10^5 to 10^{12} copies/mL	Arruda et al., 2019
Brain tissues	Weak-born neonatal piglets	From 10^{11} to 10^{12} copies/mL	
Lung tissues	PDNS / acute deaths of weaned piglets	Mean of 10^8 copies/mL	
Serum	NA	From $10^{2.5}$ to $10^{6.7}$ copies/mL	Wozniak et al., 2020
Fecal		From $10^{2.5}$ to $10^{6.0}$ copies/mL	
Oral fluids		From $10^{2.5}$ to $10^{7.2}$ copies/mL	
Fetal or stillborn piglet tissues		From $10^{3.1}$ to $10^{10.4}$ copies/mL	
Tissues from mummified piglet	Reproductive failure cases	10^9 copies/mL	Vargas-Bermúdez et al., 2021

The viral load unit of measurement from Faccini et al. (2017), Klaumann et al. (2018a), and Klaumann et al. (2019) was transformed from µL to mL to compare values among studies, NA: Non-available in the published manuscript; BQL: below the quantification limit of the technique.

1.4.7 Laboratory techniques to detect PCV-3

Development of laboratory tools to detect an infectious agent is crucial to study its potential association with disease as well as to better understand the epidemiology of the infection. Such techniques can be divided in detection of the agent or its genetic material and of the antibodies against the agent. Among the first ones, the most used methods are molecular techniques such as PCR and qPCR (Table 1.13). Other techniques such as Sanger sequencing and NGS can also be used for phylogenetic studies (Phan et al., 2016; Palinski et al., 2017; Ku et al., 2017; Franzo et al., 2018c; Fux et al., 2018; Ye et al., 2018; Yuzhakov et al., 2018; Wen et al., 2018; Arruda et al., 2019; Guo et al., 2019; Wang et al., 2019).

The serological tests described so far are very limited. Palinski et al. (2017) developed an indirect ELISA (iELISA) test using a purified recombinant PCV-3 *cap* protein to coat the plate. With this test, a prevalence of PCV-3 antibodies of 56.6% (47/83) in samples from multiple states was found (Palinski et al., 2017). Similarly, another ELISA with recombinant PCV-3 *Cap* protein was developed by Deim et al. (2018) which found prevalence of 22.3% (76/370) and 51.9% (152/293) in China from samples taken in 2015 and 2017, respectively. Recently, Mora-Díaz et al. (2020) developed two types of iELISA (an iELISA using the whole PCV-3 isolate to coat the plates and a PCV-3 recombinant *Cap* protein iELISA) and an indirect immunofluorescence assay (iIFA). The mentioned tests were developed to evaluate the reactivity of anti-PCV3 polyclonal antibodies developed in mice and also to verify the antibody response of the animals experimentally inoculated with a PCV-3 isolate, respectively (Mora-Díaz et al., 2020). Despite the usefulness of these tests enabling herd serological surveys, there is no commercial kits available, so, antibody detection techniques have been exclusively used for scientific purposes and by a minimal number of research groups worldwide.

Techniques such as ISH and IHC have been developed to detect PCV-3 genome or antigen, respectively, in FFPE tissues. The ISH technique detects the viral genome in tissues using a probe to localize the genetic material (Kedkkovid et al., 2018). Nowadays a new technology of ISH named RNAscope is also available and widely used to target PCV-3 (Phan et al., 2016; Arruda et al., 2019; Mora-Díaz et al., 2020; Vargas-Bermúdez et al., 2021). This technique targets the viral mRNA allowing the localization of viral replication in cells and improving the signal of the stained infected ones.

Table 1.13. Techniques used/developed to detect PCV-3 genome, antigen or its antibodies.

Technique	Target	Primer and probe sequence (5'-3')	Reference
Conventional PCR	Rep gene	F - TGCTACGAGTGTCCTGAAGAT R - CTTCTCCGCAACTTCAGTCAG	Fu et al., 2017
		F - AAAGCCCGAAACACAGGTGGTGT R - TTTTCCCGCATCCTGGAGGACCAAT	Franzo et al., 2018b
		F - CGAGAATTCCGAGATTGGCGAAGATTCC R - CGAGAATTCTCTCGAGGTAAT CCCCCTCT	Ye et al., 2018
	Cap gene	F - GGGCACACAGCCATAGAT R - TTCCGGGAC ATAAATGCT	Chen et al., 2017
		F - TTACTTAGAGAACGGACTTGTAACG R - AAATGAGACACAGAGCTATATTCAG	Ku et al., 2017
		F - GTGCCGTAGAAGTCTGTCATT R - TACACTCAGCCCTGTAATTTCT	Sun et al., 2017
		F - CGAGAATTCGCATAAGGG TCGTCTTGGAG R - CGAGAATTCTATGCG GAAAGTTCCACTCG	Ye et al., 2018
		F - ATGAGACACAGAGCTATATT R - TTAGAGAACGGACTTGTAAC	Guo et al., 2019
		F - TTACTTAGAGAACGGACTTGTAACG R - AAATGAGACACAGAGCTATATTCAG	Wang et al., 2019

Table 1.13. (Continuation) - Techniques used/developed to detect PCV-3 genome, antigen or its antibodies.

qPCR	Taqman	Cap gene	F - AGT GCT CCC CAT TGA ACG R - ACA CAG CCG TTA CTT CAC Probe- FAM–ACCCCATGG–Zen- CTCAACACATATGACC– BHQ1	Palinski et al., 2017
		Rep gene	F - TGACGGAGACGTCGGGAAAT R -CGGTTTACCCAACCCCATCA- Probe -FAM-GGGCGGGGTTTGCGTGATTT-BHQ1	Franzo et al., 2018b
	SYBER Green	Cap gene	F - GTGCCAGGG CTTGTTATTCT R - CTATTCATTAGGAGGCCCACAG	Jiang et al., 2019
			F - AACGGTGGGGTCATATGTGTTG R - AGACGACCCTTATGCGGAAA	Tochetto et al., 2020

Table 1.13. (Continuation) - Techniques used/developed to detect PCV-3 genome, antigen or its antibodies.

Multiplex qPCR	Taqman	Cap gene of PCV-3 and PCV-2	PCV-3 F - CGGTGGGGTCATATGTGTTG PCV-3 R - CACAGCCGTTACTTCACC PCV-3 Probe - ROX-CTTTGTCCTGGGTGAGCGCTGGTAG-BHQ2 PCV-2 F - CCAGGAGGGCGTTSTGACT PCV-2 R - CGYTACCGYTGGAGAAGGAA PCV-2 Probe - FAM-AATGGCATCTTCAACACCCGCCTCT-TAMRA	Kim et al., 2017
		Cap gene of PCV-3 and Rep gene of PCV-2	PCV-3 F - GGTGAAGTAACGGCTGTGTTTT PCV-3 R - ACACTTGGCTCCARGACGAC PCV-3 Probe -FAM-ATGCGGAAAGTTCCACTCGK-BHQ1 PCV-2 F- GARACTAAAGGTGGAACTGTACC PCV-2 R- TCCGATARAGAGCTTCTACAGC PCV-2 Probe - VIC-AGGAGTACCATTCCAACGGGG-BHQ1	Wang et al., 2019
	SYBER Green	Rep gene of PCV-3 and Cap gene of PCV-4	PCV-3 F - CGACCGAGTGGGAATCTA PCV-3 R - AGGCATCTTCTCCGCAAC PCV-4 F - CCACATAGTCTCCATCCAGTTG PCV-4 R - TACAGCCTCCCATTTGCATATTA	Hou et al., 2021

Table 1.13. (Continuation) - Techniques used/developed to detect PCV-3 genome, antigen or its antibodies.

iELISA	MAb	Anti-Cap antibody	NA	Palinski et al., 2017
	Type of Ab NI	Anti-Cap antibody	NA	Deng et al., 2018
	PAb	IgM / IgG	NA	Mora-Díaz et al., 2020
iIFA	PAb	Anti-PCV-3 whole viral particle antibody	NA	Mora-Diaz et al., 2020
IHC	MAb	Antigen (Cap protein)	NA	Palinski et al., 2017;
	MAb	Antigen (Cap protein)	NA	Li et al., 2018
ISH	RNAscope	mRNA	RNAscope probe (catalog number not informed)	Phan et al., 2016
			RNAscope probe (catalog number 463961 or 530431)	Arruda et al., 2019
			RNAscope probe (catalog number not informed)	Mora-Díaz et al., 2020
			RNAscope probe (catalog number not informed)	Vargas-Bermúdez et al., 2021
	PCR DIG		PCR DIG Probe* (primers not provided)	Kim et al., 2018a
	PCR DIG	DNA fragment of Rep gene	PCR DIG with primers targeting ORF1: 1 - ATACTGCAGGCATCTTCTCCG 2 - TATTGTGGAGTGTGGAGGCAGT	Kedkovid et al., 2018b

NA: Not applicable; MAb: Monoclonal Antibody; NI: Not informed in the published manuscript; PAb: Polyclonal Antibody; * Synthesis Kit (Roche Diagnostics, Germany).

On the other hand, the IHC is used to detect viral antigen infected cells. Palinski et al. (2017) described an IHC with an in house developed specific monoclonal antibody (MAb) for PCV-3 *Cap* protein. Later on, Li et al. (2018) also produced a PCV-3 *Cap* protein specific MAb to standardize the IHC technique on FFPE PCV-3 qPCR positive lymphoid tissues. However, none of these two IHC techniques developed offer clear-cut and conclusive results on viral detection in tissues.

1.5 Porcine circovirus 4

In 2019, a putative novel PCV with 66.9% of nucleotide identity with Mink circovirus (MiCV) was firstly described in China (Zhang et al., 2019). The virus was detected in sera and pooled tissues of pigs displaying respiratory and digestive clinical signs as well as in animals displaying PDNS-like lesions. All tested samples were negative to PCV-3 and one out of five serum samples was positive to the newly proposed PCV and PCV-2. The novel circovirus showed 50.3, 51.5 and 43.9 % of nucleotide identity with the already known PCV-1, PCV-2 and PCV-3, respectively, and was named as PCV-4 (Zhang et al., 2019). Although the new virus was found in clinical samples, the pathogenesis of PCV-4 remains unknown. Another two Chinese studies reported the virus in a frequency of 25.4% (16/63) in diseased animals (clinical signs not indicated in the paper) (Tian et al., 2020), and in 5.1% (13/257) of pigs with PDNS-like signs (Sun et al., 2021). Recently, in South Korea, the new virus has been also found in pooled organs from healthy and sick animals in a frequency of 3.28% (11/335) (Nguyen et al., 2021). On the contrary, PCV-4 DNA was not detected in serum and tissue samples from pigs of Spain and Italy (Franzo et al., 2020b).

CHAPTER 2
HYPOTHESIS AND OBJECTIVES

Hypothesis and objectives

At the beginning of the present PhD Thesis (2017), PCV-3 disease causality was unclear and the evidence pointing out such association was minimal. The first detection of PCV-3 genome corresponded to animals displaying a variety of clinical signs such as respiratory and neurological signs, reproductive failure, a PDNS-like condition and also cardiac and multisystemic inflammation (Phan et al., 2016; Palinski et al., 2017). Subsequently, further reports also indicted potential association of PCV-3 with digestive clinical signs (Zhai et al., 2017; Qi et al.,2019), respiratory disorders (Zhai et al., 2017; Kedkovid et al., 2018b; Shen et al., 2018; Qi et al.,2019), reproductive problems (Faccini et al., 2017; Deim et al., 2019; Arruda et al., 2019) and multisystemic inflammation (Arruda et al., 2019).

Most of these studies with diseased animals did not test age-matched healthy animals as control groups to compare with. In fact, during these last years, subclinical infection in healthy animals has been reported by many authors (Stadejek et al., 2017; Zhai et al., 2017; Klaumann et al., 2018a; Klaumann et al., 2018b; Klaumann et al., 2019; Franzo et al., 2018; Ye et al., 2018; Saporiti et al., 2020), so, it is considered that PCV-3 is probably widespread all over the world. In addition, one should bear in mind that the simple viral detection in animals with clinical and pathological manifestations without showing evidence of viral presence in the corresponding lesions is not sufficient to establish pathogen causality. In fact, much more studies under both experimental and natural conditions are required to definitively establish PCV-3 as a true pathogen.

Thus, the present Thesis aimed to gain knowledge on PCV-3 disease association by studying its detection in pigs suffering from different clinical-pathological conditions. The specific objectives were:

- To evaluate the frequency of PCV-3 in diseased animals displaying well characterized histologic lesions of respiratory and digestive diseases and compare to the frequency of viral detection in healthy animals (Chapter 3, Study I)
- To describe the normal percentage of PCV-3 infection in primiparous and multiparous sows and in their respective mummified and stillborn piglets from farms without overt reproductive problems (Chapter 4, Study II)
- To assess the frequency of PCV-3 detection in cases of reproductive problems from Spanish farms and its possible association with the lesions observed in aborted fetuses (Chapter 5, Study III)

Hypothesis and objectives

- To describe the presence of PCV-3 nucleic acid within the lesions of post-weaning pigs affected by a multisystemic inflammatory condition (Chapter 6, Study IV).

CHAPTER 3

STUDY I

Similar frequency of *Porcine circovirus 3* (PCV-3) detection in serum samples of pigs affected by digestive or respiratory disorders and age-matched clinically healthy pigs

Transboundary and Emerging Diseases. 2020, 67: 199–205.
DOI: 10.1111/tbed.13341

Study I – PCV-3 in respiratory and digestive disorders

3.1 Introduction

Since the very first description of PCV-3, this virus has been found in pigs with respiratory clinical signs and lung lesions (Zhai et al., 2017; Kedkovid et al., 2018b; Shen et al., 2018; Qi et al., 2019), digestive disorders (Zhai et al., 2017; Qi et al., 2019), congenital tremors (Chen et al., 2017), rectal prolapse (Phan et al., 2016) and peri-weaning failure-to-thrive syndrome (Franzo et al., 2019). However, several of these studies where PCV-3 was found in diseased animals failed to provide healthy age-matched pigs as control groups (Kedkovid et al., 2018b, Palinski et al., 2017, Phan et al., 2016, Shen et al., 2018).

Between 2017 and 2019, two studies found PCV-3 DNA in pigs with severe and/or mild respiratory disease compared to healthy animals, suggest a potential involvement of this virus in disease causation (Zhai et al., 2017; Qi et al., 2019). Same studies also suggested a putative association with digestive disorders based on the higher frequency of detection of the virus DNA in diseased animals compared to healthy ones (Zhai et al., 2017; Qi et al., 2019).

Taking into account the abovementioned reports, the present study aimed to evaluate the putative association of PCV-3 infection in well-pathologically characterized cases of pigs suffering from respiratory or digestive disorders in comparison to age-matched clinically healthy animals.

3.2 Material and methods

3.2.1 Samples

The study was performed with 255 swine serum samples obtained from animals affected by digestive or respiratory disorders necropsied for diagnostic purposes. These samples were stored at -20ºC at the serum bank of the Veterinary Pathology Diagnostic Service at the Veterinary School of Barcelona. The selection criteria of these animals were:

1) age (animals from 1-4 months of age (nursery and grower pigs), and 2) presence of histopathological lesions at the respiratory (n=129) or enteric (n=126) tracts. A negative control group of clinically healthy animals were selected for comparison purposes. A total of 60 sera were retrieved from the serum bank of the *Centre de Recerca en Sanitat Animal* (CReSA-IRTA), and consisted in a selection of necropsied (n=30) and alive (n=30) 1-4 month-old pigs with no clinical signs and no lesions. The necropsied animals belonged to control groups of different CReSA experimental inoculation studies. The clinically healthy live pigs came from previous CReSA field trials. All selected samples (n=315) were analysed for the presence of PCV-3 DNA.

Pigs affected by respiratory clinical signs **(**group R**)** were classified regarding specific gross and/or microscopic findings (Table 3.1). Animals with interstitial pneumonia (IP, n=30), suppurative bronchopneumonia (SBP, n=30), IP plus SBP (IP+SBP, n=30), pleuritis (P, n=19) and fibrinous-necrotizing pneumonia (FNP, n=20) were selected. Animals with digestive disorders **(**group D**)** were also classified by histopathological findings, including pigs with catarrhal enteritis (CE, n=55), CE with villi atrophy and fusion (CE+AFV, n=25) and catarrhal colitis (CC, n=46). The control group was composed of sera from 60 clinically healthy animals **(**group H).

3.2.2 DNA extraction, and conventional and quantitative PCR methods

DNA was extracted from 200 µL of serum using MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol.

A conventional PCR targeting the *rep* gene region (ORF1) of PCV-3 was designed. Each reaction included 2.5 µL of extracted DNA, 12.5 µL of Go Taq® G2 Green Master Mix (Promega), 0.4 µM of each primer (forward (P1F) 5`-TTGTGGTGCTACGAGTGTCC-3` and reverse (P1R) 5`-

CGTCTCCGTCAGAATCCGAG-3`), and sterile water at a final volume of 25 µL. Amplification was performed using the following thermal conditions: 5 min at 94˚C, followed by 40 cycles of 1 min at 94˚C, 1 min at 60˚C and 1 min at 72˚C, and a final elongation of 7 min at 72˚C. A full-length PCV-3 genome in a commercial plasmid (Klaumann et al., 2018c) was used as positive control and sterile water as negative control. The PCR products (418 bp) were checked by electrophoresis on 1.5% TAE agarose gel.

A real time quantitative PCR (qPCR) to quantify the amount of viral DNA of conventional PCR positive samples was performed as previously described (Franzo et al., 2018a), with slight modifications. Briefly, 2 µL of extracted DNA was added to a mix of 1xDyNAmo Colour flash Probe qPCR master mix (Thermo Fisher Scientific), 0.6 µM and 0.3 µM of primers and probe targeting PCV-3 virus, 0.6 µM and 0.3 µM of primers and probe for internal control (IC), and 1 pg of IC plasmid (Klaumann et al., 2018c). The qPCR was performed with Applied Biosystems® 7500 Real-Time machine with cycling parameters of 95˚C for 7 min, followed by 40 cycles of 95˚C for 10s and 60˚C for 30s. Sterile water was used as negative controls. A tenfold log dilution of the commercial plasmid mentioned above was used to construct the standard curve. The qPCR results were expressed in copies of PCV-3 DNA/mL of serum.

3.2.3 PCV-3 sequencing and phylogenetic studies

Two different sequencing strategies based on *rep* gene and complete genome were attempted.

Firstly, to partially sequence the *rep* gene (362 nt), the conventional PCR primers were used; conditions for amplification included the utilisation of the DNA polymerase Platinum™ SuperFi™ (Invitrogen™) kit and the thermal protocol of 2 min at 95˚C, followed by 40 cycles of 30 s at 95˚C, 1 min at 60˚C and 1 min at 72˚C, and a final elongation of 7

min at 72˚C. The amplicons were purified with a Kit NucleoSpin® Gel extraction (Macherey-Nagel) according to the manufacturer's protocol and sequenced by the Sanger method (ABI 3730XL - Macrogen Europe, Madrid, Spain). The quality of the sequences was analysed by the Finch TV program and trimmed in BioEdit software 7.2.6 (Hall, 1999) together with all the sequences containing the same fragment available in GenBank (247 sequences, accession date April 03rd, 2019). Clustal Omega (Sievers and Higgins, 2014) was used to align and generate the percentage of nucleotide identity matrix with the obtained sequences. Sequences were deposited at the NCBI GenBank with the accession numbers MK904813 to MK904827.

Secondly, with the aim to sequence the full genome from PCV-3 PCR positive samples, a rolling circle amplification (RCA) method was attempted to increase the amount of PCR template using the ThempliPhi 100 amplification Kit (GE Healthcare) as described by Fux et al. (2018). For the PCR, 3 µL of the 1:10 diluted RCA product was used to amplify the complete PCV-3 genome using previously described primers (Fux et al., 2018) and thermal conditions (Klaumann et al., 2018c). The amplicons were purified as described above and subjected to Sanger sequencing (ABI 3730XL - Macrogen Europe, Madrid, Spain). The raw chromatograms were manually inspected with Finch TV and the trimming was done with the BioEdit software 7.2.6 (Hall, 1999). Partial PCV-3 genome sequence assembly was done with the different amplicons using reference mapped-based strategy (Li and Durbin, 2010). The Integrative Genomics Viewer (IGV) software (Robinson et al., 2017) was used for visualizing the assembly and obtaining the consensus sequences for ORF1 and ORF2. Concatenated *rep* and *cap* genes obtained were 1237 nt in length and were subsequently aligned with the 43 sequences within the same genomic region previously used and classified by Fux et al. (2018) using Muscle (Edgar, 2004) integrated in MEGA 7 (Kumar et al., 2016). A maximum likelihood (ML) tree was constructed using the

substitution model test with the lowest BIC score (MEGA7). The Tamura-Nei model was used to build the phylogenetic tree, with bootstrapping at 1000 replicates to analyse the robustness of the clustering. The tree was edited using the iTOL program (Letunic and Bork, 2019). Sequences were deposited at the NCBI GenBank with the accession numbers MK904828 to MK904831. The already suggested PCV-3 subgrouping classification (PCV-3*a1*, PCV-3*a2,* PCV-3*b1* and PCV-3*b2,* Fux et al., 2018) was done regarding the amino acid (aa) motifs from *Rep* and *Cap* proteins.

3.2.4 Statistical analysis

The Clopper-Pearson method (Clopper and Pearson, 2006) was used to calculate the exact confidence interval (CI) at 95% level of confidence for the obtained PCV-3 PCR positive frequencies. The frequency of PCV-3 DNA detection among the different groups (R, D and H) and types of lesions within each group was compared with the Chi-square or Fisher´s exact tests using GraphPad software (GraphPad software Inc., San Diego, USA). P-value <0.05 were considered statistically significant.

3.3 Results

3.3.1 Detection of PCV-3

PCV-3 DNA was found in 19 out of the 315 studied samples (6.0%, 95% confidence interval, CI: 3.7-9.3). The frequency of detection was 6.2% (8 out of 129, 95% CI: 2.7-11.9) in pigs from group R and 5.6% (7 out of 126, 95% CI: 2.3-11.1) in pigs from group D. Viral DNA was also detected to a similar frequency in group H, being 4 out of 60 (6.7%, 95% CI: 1.8-16.2) PCV-3 PCR positive healthy pigs (Table 3.1). No statistical differences were observed among studied groups, and no association of any specific lesion with PCV-3 detection was found.

Conventional PCR positive samples were quantified by qPCR, except five samples that were below the quantification limit of the technique (10^3 of DNA/mL). The PCV-3 load detected in the positive cases was low, with mean viral loads in the different groups of lesions ranging from $10^{3.5}$ to $10^{5.8}$ copies/mL of serum (Table 3.1).

Table 3.1. Number and percentage of PCV-3 PCR positive serum samples and PCV-3 viral load range in pigs displaying respiratory and digestive lesions as well as in healthy pigs.

Groups	Lesion	Number of samples (n)	PCV−3 positive PCR (n)	Percentage of PCR positivity	PCV−3 copies/mL (mean)
Respiratory	IP	30	3	10.0	$10^{4.3}$
	SBP	30	2	6.6	$10^{5.8}$
	IP+SBP	30	2	6.6	$10^{3.5}$
	P	19	1	5.2	BLQ
	FNP	20	0	0.0	-
	Sub-total	129	8	6.2	$10^{4.5}$
Digestive	CE	55	3	5.4	$10^{3.8}$
	CE+AFV	25	2	8.0	10^4
	CC	46	2	4.3	$10^{3.9}$
	Sub-total	126	7	5.5	$10^{3.9}$
Healthy	Sub-total	60	4	6.6	$10^{4.3}$
Total		315	19	6.0	$10^{3.9}$

IP, interstitial pneumonia; SBP, suppurative bronchopneumonia; P, pleuritis; FNP, fibrinous-necrotizing pleuropneumonia; CE, catarrhal enteritis; AFV, atrophy and fusion of villi; CC, catarrhal colitis; BLQ, under the limit of quantification of the qPCR.

3.3.2 Sequence and phylogenetic analysis

From the 19 PCR positive samples, 15 sequences from the *rep* gene fragment were obtained. From these sequences, eight belonged to pigs from the group R, three from group D and four from group H. The identity matrix comparing these 15 *rep* fragments showed a percentage of identity ranging from 98.34 to 100%. When these fragments were compared with the same genomic regions of 247 available GenBank reference sequences, such percentage ranged from 91.99 to 100%.

Four sequences of 1237 nt in length (including the complete *rep* gene with 890 nt and part from the *cap* gene with 347 nt) were obtained from four RCA-PCR positive samples: two from samples belonging to group H (samples No. 409 and 441); one from group R (sample No. 1099) and one from group D (sample No. 169). Based on the ML phylogenetic tree, samples No. 409 and 1099 belonged to the proposed subgroup *a1*, while samples No. 441 grouped into subgroup *b1* and No. 169 were from subgroup *b2* (Figure 3.1).

3.4 Discussion

The present study aimed to expand the current knowledge on PCV-3 respiratory and digestive disease association based on a case-control study performed with clinical-pathologically well-characterized pigs. Each analysed group corresponded to specific pathological findings that are generically considered to be caused by bacteria (SBP, P, FNP, CE, CC) or viruses (IP, AFV+CE) (Caswell and Williams, 2016; Uzal et al., 2016).

In contrast to the two previous studies evaluating such putative relationship (Zhai et al., 2017; Qi et al., 2019), no significant differences in terms of PCV-3 DNA frequency detection were found among pigs with respiratory or digestive disorders compared between them or with age-matched healthy animals.

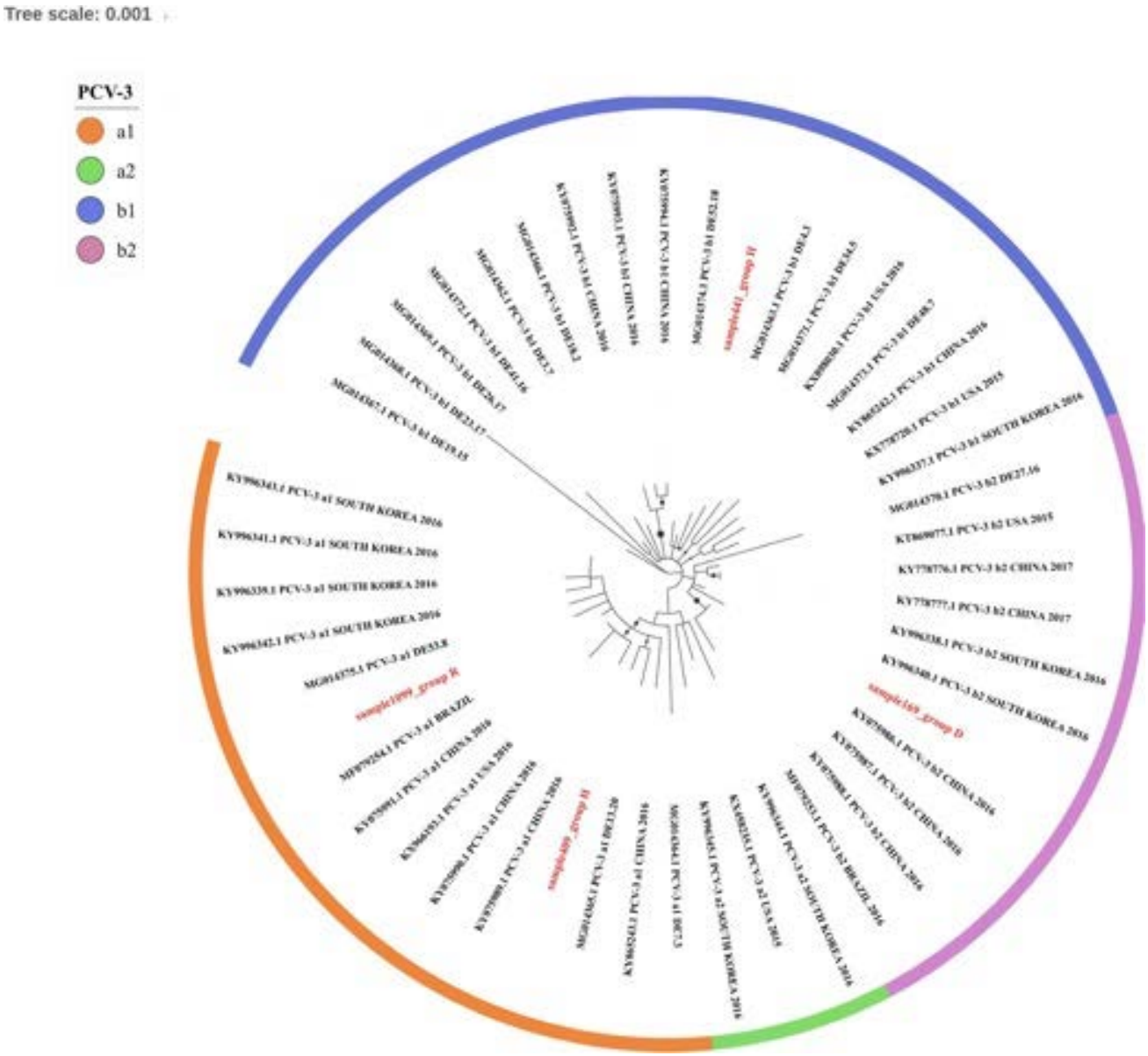

Figure 3.1. Phylogenetic analysis of PCV-3 based on partial genome (1,237 nt). The tree was constructed using maximum likelihood method (Tamura-Nei model) at 1,000 bootstrap. Circles in branches represent bootstrap values with sizes proportional to the bootstrap value (only values higher than 50 are shown). Sequences obtained in the present study are labelled in red. Colours indicate the subtypes of PCV-3 suggested by Fux et al. (2018).

The previously mentioned studies referred to samples from China and used either serum (Zhai et al., 2017) as in the present work or intestinal/lung tissues (Qi et al., 2019), collected based exclusively in the animals showing clinical signs. The matrix (sample type) used for studying PCV-3 frequency of infection may offer some variability (Phan et al., 2016; Klaumann et al., 2018c; Shen et al., 2018) and, therefore, may partly explain differing results between the present study and that of Qi et al. (2019). However, the same potential explanation would not apply to the other study (Zhai et al., 2017), since serum was also used from affected and non-affected pigs.

The two abovementioned works studying the frequency of PCV-3 in pigs with digestive disorders also included control animals. Specifically, Qi et al. (2019) used intestinal tissue samples from 480 pigs with acute diarrhoea and/or vomiting and 42 faeces of healthy pigs; whereas 50 out of 480 samples (10.4 %) were PCV-3 PCR positive, all 42 healthy pigs were found to be negative. On the other hand, Zhai et al. (2017) found 17.14 % (6/35) PCR positive serum samples from pigs with diarrhoea and 2.86 % (1/35) in animals without clinical signs. Both studies also found significant differences when comparing pigs affected by respiratory problems and healthy ones. Indeed, Qi et al. (2019) found 26.6% (25/94) and 0% (0/42) PCV-3 frequencies in diseased and healthy animals, respectively. Similarly, Zhai et al. (2018) found a PCV-3 PCR positivity of 29% (split as 63.75%, 51/80, in pigs with severe respiratory disease and 13.14%, 23/175, in animals with mild signs) and 1.9% (4/216) in healthy pigs, respectively. In another study, pigs with respiratory disease complex (PRDC) from Thailand showed significantly higher PCV-3 detection rate (60%, 15/25) in serum compared to healthy pigs (28%, 7/25) (Kedkovid et al., 2018b). The apparently contradictory results offered by the present study may represent different epidemiological situations in different countries and/or different sensitivity of PCR techniques used.

Noteworthy, animal selection criteria from previous studies were mainly based on clinical signs. For this reason, the present work split the clinical conditions by observed lesions in each case. This selection is important, since lesions like IP and AFV+CE are generally attributable to viral infections (Caswell and Williams, 2016; Uzal et al., 2016), but no higher frequency of PCV-3 detection was found in any group of animals displaying these pathological findings. In consequence and supported by the fact that very low viral loads were found in analysed animals, obtained results do not point out to PCV-3 as a likely cause of these lesions. In contrast, two of the abovementioned Asian studies (Zhai et al., 2017; Kedkovid et al., 2018b) indicated that animals displaying respiratory clinical signs had higher viral load than healthy ones. In the study from Zhai et al. (2017), pigs with severe respiratory disease showed Ct values lower than 30 while the ones with either mild respiratory disease or healthy animals showed Ct values higher than 25 or 30, respectively (Zhai et al., 2017). The sick pigs from Kedkovid et al. (2018b) study presented titres of 3.2 ± 2.8 log genomic copies/mL, while healthy ones had lower titres (1.6 ± 2.6 log genomic copies/mL). In all cases, it would have been interesting to assess the type of lesions those diseased animals displayed in these studies.

The relatively low frequency of PCV-3 DNA detection in all pig groups fits relatively well with a previous retrospective study performed in Spain (Klaumann et al., 2018a) where 11.4% (75 out of 654) of PCV-3 serum samples were found PCR positive. Nevertheless, and based on existing literature, the prevalence of PCV-3 infection seems to be very variable (Klaumann et al., 2018b). This fact makes difficult to establish if such differences are due to a real variability among countries or simply reflect the frequency of a limited number or type of samples examined in each study.

The phylogenetic analysis of all obtained partial sequences (the ones with 362 nt as well as those with 1237 nt) showed high percentage of nucleotide identity compared to the

publicly available sequences, as it has been already published for this virus (Zheng et al., 2017; Fux et al., 2018; Franzo et al., 2018a; Zhou et al., 2018). Although Fux et al. (2018) proposed different subtypes for PCV-3, the very high nucleotide identity of available sequences for this virus do not suggest a potential relation with the clinical or pathological outcome; in fact, no clustering of the obtained sequences was observed regarding the disease status.

In summary, PCV-3 DNA was found to similar percentages in pigs affected by both respiratory and enteric disorders as well as in age-matched healthy animals. Moreover, no apparent association was found between the presence of viral genome in serum and particular lesions generally attributed to viral infections in both respiratory and digestive tracts. Therefore, the results obtained throughout this study do not support a potential association of PCV-3 with respiratory or enteric disease occurrence. Definitively, further studies are needed to elucidate the putative association of PCV-3 with different pathological outcomes.

CHAPTER 4

STUDY II

Frequency of detection and phylogenetic analysis of *Porcine circovirus 3* (PCV-3) in healthy primiparous and multiparous sows and their mummified and stillborn fetuses

Pathogens. 2020, 9:1–12.
DOI: 10.3390/pathogens9070533

Study II – PCV-3 detection in animals from farms without reproductive disorders

4.1 Introduction

The PCV-3 genome was initially found in cases of reproductive disorders, specifically in mummified fetuses and abortions (Palinski et al., 2017). Although PCV-3 pathogenesis is poorly known, a high number of reports have pointed out a potential causality association of PCV-3 with reproductive disease based on virus detection and clinical signs in the absence of other pathogens (Phan et al., 2016; Palinski et al., 2017; Kim et al., 2018a; Tochetto et al., 2018; Zou et al., 2018; Deim et al., 2019; Dal Santo et al., 2020). This putative association is also reinforced by a newly released study where the virus was successfully isolated from cases of reproductive losses (Mora-Díaz et al., 2020).

The PCV-3 genome is composed of 1999–2001 nt (Fux et al., 2018; Ha et al., 2018) with two well-characterized ORFs, ORF1 encoding the Rep and ORF2 encoding the Cap. ORF1 and ORF2 are in positive and negative strands, respectively (Phan et al., 2016; Palinski et al., 2017). Although being from the same family, PCV-2 and PCV-3 are far different in terms of amino acid (aa) homology, sharing only 48% of identity in the Rep protein (Phan et al., 2016) and between 26% and 37% in the Cap protein (Phan et al., 2016; Palinski et al., 2017). Despite PCV-3 available sequences sharing high similarity among them, different classification systems based on aa marker positions have divided PCV-3 into two (PCV-3a and PCV-3b) (Fux et al., 2018; Li et al., 2018) or three (PCV-3a, PCV-3b, and PCV-3c) (Fu et al., 2018) main groups. However, a recent study highlighted several exceptions for the mentioned marker positions and proposed a definition based on only one PCV-3 genotype to date, the PCV-3a (Franzo et al., 2020a).

Considering the potential association of PCV-3 with reproductive cases, the objective of this study was to assess the frequency of detection of PCV-3 and phylogenetically analyze the virus in serum samples from primiparous and multiparous sows from farms without reproductive problems, as well as in tissues from the respective mummified or stillborn piglets. This study was performed to establish the "PCV-3 infection background" in normally performing farms.

4.2 Material and methods

4.2.1. Samples

Sera from 121 sows belonging to 3 different farms (A, n=44; B, n=37; and C, n=40) were collected at two time points; the first one (S1) close or at pre-mating and the second sampling (S2) close or at farrowing time. These farms showed good reproductive parameters

as farms A, B, and C presented means of 0.38, 0.29, and 0.56 mummies out of 14.39, 14.15, and 14.79 mean of piglets born per litter, respectively. These values are considered even lower than the average of the mummies/stillborn of Spanish pig farms included in the BDPorc database (http://www.bdporc.irta.es/index.jsp). All farms were conventional ones, seropositive against porcine reproductive and PRRSV, PPV, and PCV-2, but negative to ADV. The normal vaccination schedule included PRRSV and PPV/erysipelas vaccination of sows by cycle, as well ADV vaccination in a blanket fashion. Gilts were vaccinated against PRRSV and PPV/erysipelas during the acclimation period. The performed study was approved by The Zoetis Olot Animal Welfare Committee prior to the start of the experiment, with reference number 382, and it was notified to and approved by Spanish Authorities.

From the 121 total sampled sows, 57 were primiparous and 64 were multiparous (≥ second parity) (Table 4.1). All dams, primiparous and multiparous, were sampled at two time points (S1 and S2). Additionally, tissues (brain and lung) from a total of 255 mummified or stillborn piglets from the respective sampled sows were also included in this study, except for primiparous ones from farm B, from which the fetuses were not available for the study (Table 4.2). The number of collected fetuses ranged from 1 to 18 animals per litter. The time of gestation at which the fetuses died was determined by its body size at delivery and physical aspect (Althouse et al., 2019). Thus, by the physical aspect, the fetuses were classified as mummified (n=49) or stillborn (n=206) (Muirhead et al., 2013). Noteworthy, all fetuses (mummified and stillborn) were collected at the expected farrowing time.

4.2.2. DNA extraction, PCR and qPCR

Tissue samples from the fetuses (brain and lungs) were homogenized separately. DNA extraction from 200 µL of macerated tissue supernatant as well as 200 µL of sera from sows was performed using a MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol.

A conventional PCR targeting the PCV-3 rep gene region (ORF1) was performed as previously described (Study I). The PCR products were checked by electrophoresis on 1.5% TAE agarose gel.

To quantify the amount of virus in the PCR positive samples, a real-time quantitative PCR (qPCR) was performed also as previously described (Franzo et al., 2018b; Study I). The qPCR results were expressed in log10 of PCV-3 DNA copies/mL of serum or supernatant of macerated tissues sample.

4.2.3. PCV-3 phylogenetic analysis

Those qPCR positive samples with the highest amount of virus in both fetal tissue supernatant (from 5.74 to 10.84 log10 copies/mL) and sow serum samples (from 3.88 to 4.91 log10 copies/mL) were selected to be sequenced by means of PCRs amplifying the whole PCV-3 genome (Fux et al., 2018). When possible, samples from sows and fetuses from the same litter (with a high amount of virus) were selected. The PCR reaction contained 1 × PCR buffer, 0.4 µM of dNTPs, 0.2 µM of each primer, 1 Unit of DNA polymerase Platinum™ SuperFi™ (Invitrogen™), and water to bring the final volume up to 50 µL. The thermal conditions included 98 °C for 5 min followed by 40 cycles of 98 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min, plus the final elongation at 72 °C for 7 min. The obtained amplicons were purified with an ExoSAP-IT® Express PCR product Cleanup (Thermo Fisher Scientific) Kit according to the manufacturer's protocol and sequenced by the Sanger method (ABI 3730XL - Macrogen Europe, Madrid, Spain). The quality of the sequences was analyzed by the Finch TV software and trimmed in BioEdit vs. 7.2.6 (Hall et al., 1999). Obtained amplicons were assembled using the reference mapped-based strategy (Li and Durbin, 2010) to achieve PCV-3 complete genomes. The Integrative Genomics Viewer software (Robinson et al., 2017) was used for visualizing the assembly and extracting the consensus sequence. The complete genome sequences were aligned in BioEdit vs. 7.2.6 (Li et al., 2010) with summarized collected reference samples according to a previously published method (Franzo et al., 2020a), as well as the ORF2 gene and the translated ORF2 region with the standard genetic code (using MEGAX). The best substitution method was selected based on the lowest Bayesian Information Criterion (BIC) score calculated on MEGAX software (Kumar et al., 2018), either for the complete genome analysis or for the ORF2 aa analysis. The Maximum Likelihood tree with Hasegawa–Kishino–Yano (HKY) model (Hasegawa et al., 1985) plus Gamma distribution phylogenetic was used to construct the phylogenetic tree for the complete genome and the tree for the ORF2 gene both with 1000 bootstrap replicates using MEGAX software (Kumar et al., 2018). The translated ORF2 region was used to build a Neighbor Joining (NJ) phylogenetic tree constructed using the Jones–Taylor–Thornton's model (Jones et al., 1992) with 1000 bootstraps. The identity among nucleotide sequences was compared using Clustal Omega (Sievers and Higgins, 2014).

All PCV-3 sequences generated in this study were deposited at the NCBI GenBank with the accession numbers MT350517–MT350555.

4.2.4. Statistical analysis

The frequency of PCV-3 DNA detection in serum samples from primiparous and multiparous sows was compared globally, per farm (only from primiparous) and per sampling point. The frequency of detection as well as the median PCV-3 viral load in fetal samples were analyzed considering the type of tissue (lung or brain), the type of fetus (mummies or stillborn), their dam (primiparous or multiparous), and the farm of origin. The frequencies of detection were analyzed using the Pearson's Chi-squared test or Fisher´s exact test. The median PCV-3 viral loads were compared using the Mann–Whitney test. These analyses were carried out using GraphPad software (GraphPad software Inc.) and GraphPad Prism 8, where P-value < 0.05 was considered statistically significant.

4.3 Results

4.3.1. PCV-3 detection and virus quantification

From the 121 sows included in the study, 19 (15.7%) had at least one PCV-3 PCR-positive serum sample. All these positive samples corresponded to primiparous sows (19 out of 57, 33.3%) from the three different farms (Table 4.1). From these, one was collected at sampling point 1 (S1) and 18 were collected at sampling point 2 (S2) (P-value = 0.001); the positive sample at S1 was negative at S2. The difference in frequency of positivity between S1 and S2 was highly significant (P-value < 0.0001). However, the frequency of PCV-3 PCR positivity in primiparous sows between the three farms was not significantly different. All sera from multiparous sows were negative at both sampling times.

The viral load of the positive sera from primiparous sows ranged from 3.47 to 5.30 log10 copies/mL, with the exception of one sample with a viral load below the quantification limit of the technique (10^3 of DNA/mL).

Table 4.1. Number of Porcine circovirus 3 (PCV-3) PCR positive serum samples out of the total number of tested samples (percentage) per farm and sampling point in primiparous and multiparous sows.

Farm	Sampling point	Primiparous Sows	Multiparous Sows	Total
A	**S1**	1/19 (5.3%)	0/25 (0.0%)	1/44 (2.3%)
	S2	8/19 (42.1%)	0/25 (0.0%)	8/44 (18.2%)
B	**S1**	0/17 (0.0%)	0/20 (0.0%)	0/37 (0.0%)
	S2	3/17 (17.6%)	0/20 (0.0%)	3/37 (8.1%)
C	**S1**	0/21 (0.0%)	0/19 (0.0%)	0/40 (0.0%)
	S2	7/21 (33.3%)	0/19 (0.0%)	7/40 (17.5%)

Eighty-six out of 255 (33.7%) fetuses had at least one tissue positive for PCV-3 genome detection (Table 4.2), being 41 out of 96 (42.7%) from farm A, 2 out of 48 (4.2%) from farm B, and 43 out of 111 (38.7%) from farm C. The numbers of positive fetuses from farms A and C were not statistically different between them (P-value = 0.6613), but values from farms A and C were statistically different from those of farm B (P-value < 0.0001 in both cases). These 86 fetuses positive to PCV-3 PCR came from 14 positive (all primiparous) and 31 negative dams (being 23 primiparous and 8 multiparous sows).

Globally, the number of PCV-3 PCR positive fetal samples from primiparous sows (73 out of 91, 80.2%) was significantly higher (P-value < 0.0001) than the one from multiparous ones (13 out of 164, 7.9%). These statistically significant differences were also observed in either farm A or C, when individually analyzed (Table 4.2). Additionally, the median PCV-3 load found in fetuses' tissues from multiparous sows (4.16 log10 copies/mL) was significantly lower (P-value <0.0001) when compared to the viral load in fetuses' tissues from primiparous sows (6.57 log10 copies/mL). Moreover, the viral loads were similar in positive fetuses from the same litter; however, among mummies and stillborn from the same sow, mummies tend to have higher viral load.

The number of mummies with at least one PCV-3 PCR positive sample (27 out of 49, 55.1%) was significantly higher (P-value = 0.0008) than that of the stillborn (59 out of 206, 28.6%) (Table 4.2). The total number of PCV-3 PCR-positive mummies compared to stillborn was statistically significant in farms A and C (P-value = 0.0005 and P-value = 0.0467, respectively).

Table 4.2. Number and percentage of mummified fetuses and stillborn with at least one PCV-3 PCR-positive tissue and their viral load range.

Farm	Sow Parity	Mummified Fetuses		Stillborn		Total
		PCR Positive/Total (%)	(Min-Max) log_{10} PCV-3 Copies/mL	PCR Positive/Total (%)	(Min-Max) log_{10} PCV-3 Copies/mL	
A	**Primiparous**	13/15 (86.7%)	3.66–9.48	19/26 (73.1%)	3.56–9.66	32/41 (78.0%) ^{A}
	Multiparous	0/0 (0.0%)	-	9/55 (16.4%)	3.15–4.96	9/55 (16.4%) ^{B}
B *	**Multiparous**	0/10 (0.0%)	-	2/38 (5.3%)	4.67–5.26	2/48 (4.2%)
C	**Primiparous**	13/15 (86.7%)	3.94–10.84	28/35 (80.0%)	3.30–9.46	41/50 (82.0%) ^{A}
	Multiparous	1/9 (11.1%)	4.01	1/52 (1.9%)	3.56	2/61 (3.3%) ^{B}
Total		27/49 (55.1%) ^{a}	3.66–10.84	59/206 (28.6%)^{b}	3.15–9.66	86/255 (33.7%)

***** No fetuses from primiparous sows were available from farm B. Different letters in superscript in a row mean statistically significant differences between the total of mummies and stillborn (P-value < 0.05). Different letters different letters in upper case in a column mean statistically significant differences between fetus from primiparous and multiparous sows (P-value < 0.05).

No statistically significant differences in the median PCV-3 viral load detected in the tissues from mummies (6.56 log10 copies/mL) and stillborn (5.93 log10 copies/mL) were found.

Twenty-one out of the 27 (77.8%) and 40 out of the 59 (67.8%) PCR-positive mummies and stillborn, respectively, were positive in both tissues analyzed (lungs and brain). The frequency of PCV-3 genome detection in fetal lungs (81/255, 31.8%) was numerically higher when compared to that of the brain (66/255, 25.9%), but these differences were not significant (Table 4.3). The percentage of detection in brain and in lung was significantly higher in mummies than in stillborns in farms A and C (P-value < 0.05) (Table 4.3). The PCV-3 load ranged from 3.38 to 10.16 log10 copies/mL in brain and from 3.15 to 71084 log10 copies/mL in lungs. There were three samples from brain and three from lungs, from different animals, with viral loads below the quantification limit of the qPCR. No statistical differences in viral load were found between lung and brain tissues in fetuses neither from primiparous nor from multiparous sows.

Table 4.3. Number and percentage of PCV-3 PCR positive tissues from mummies or stillborn.

Farm	Fetuses	Tissue		Total Tissue PCR Results (%)
		Brain PCR Results (%)	Lung PCR Results (%)	
A	**Mummies**	10/15 (66.7%) [a]	13/15 (86.7%) [a]	23/30 (76.7%) [a]
	Stillborn	24/81 (29.6%) [b]	25/81 (30.9%) [b]	49/162 (30.2%) [b]
B	**Mummies**	0/10 (0.0%)	0/10 (0.0%)	0/20 (0.0%)
	Stillborn	0/38 (0.0%)	2/38 (5.3%)	2/76 (2.6%)
C	**Mummies**	11/24 (45.8%) [a]	14/24 (58.3%) [a]	25/48 (52.1%) [a]
	Stillborn	21/87 (24.1%) [b]	27/87 (31.0%) [b]	48/174 (27.6%) [b]
Total		66/255 (25.9%)	81/255 (31.8%)	147/510 (28.8%)

Different letters in superscript in a column mean statistically significant differences in PCV-3 detection between mummies and stillborn in each farm (P-value < 0.05).

4.3.2. PCV-3 phylogenetic analysis

Forty-two PCR positive samples were selected for the phylogenetic analysis, being 8 serum samples from primiparous sows, 16 tissues from mummified fetuses (8 brains and 8 lungs), and 18 tissues from stillborn (8 brains and 10 lungs). It was possible to achieve 39 complete genome sequences out of the 42 selected samples. The sequence from the remaining 3 samples (brain and lung from one stillborn and the lung from another one) showed bad quality. The nucleotide identity of these 39 samples ranged from 99.2% to 100%. When possible, PCV-3 sequences from fetuses of the same litter, as well as the respective sow, were compared. This comparison was done in four cases, and the identity of the viral sequences obtained ranged from 99.30% to 100%. Moreover, sequences from fetuses of the same litter of three other cases were compared showing an identity between 99.7% and 100%.

The phylogenetic analyses performed with the complete PCV-3 genome sequences showed two main clusters (Figure 4.1). All sequences analyzed herein belonged to Cluster 1, together with the reference sequences previously genotyped as PCV-3a (Franzo et al., 2020a). Similar results were found when only the cap region was analyzed (Figure 4.2). The overall nucleotide identity with the PCV-3a reference sequences used for the complete genome phylogenetic tree was 97.75% to 100%. When the aa sequence inferred from the ORF2 gene was phylogenetically analyzed (Figure 4.3), all samples were also classified as PCV-3a Franzo et al. (2020a), although with a different pattern of distribution among the reference sequences used. The aa identity of the inferred ORF2 protein was high, ranging from 98.9% to 100% among all the sequences obtained herein, supporting the fact that they all belong to the same cluster.

Study II – PCV-3 detection in animals from farms without reproductive disorders

Figure 4.1. Phylogenetic analysis of PCV-3 complete genome sequences. The tree was constructed for the full genome sequences obtained herein, and the PCV-3 references sequences included in Franzo et al., 2020. The best substitution model with the highest Bayesian Information Criterion (BIC) score was used with 1000 bootstraps (Hasegawa–Kishino–Yano with Gamma distribution). The width of the branches is proportional to bootstrap P-values, and the scale bar indicates nucleotide substitutions per site. The sequences obtained in this study were labeled with GenBank ID followed by farm identification, sow number and sample number; F, for fetus samples; B, for brain or L for lung tissue. Samples were colored by farm (farm A in red, farm B in blue and farm C in green).

Study II – PCV-3 detection in animals from farms without reproductive disorders

Figure 4.2. PCV-3 phylogenetic ML tree constructed with ORF2 gene using the Hasegawa–Kishino–Yano (HKY) model with Gamma distribution at 1000 bootstrap (the width of the branches is proportional to bootstrap P-values) and with GenBank reference samples included in Franzo et al., 2020a. The 39 PCV-3 ORF2 sequence samples were labeled with GenBank ID followed by farm identification, sow number, and sample number; F, for fetus samples; B, for brain; or L for lung tissue. Samples were colored by farm (farm A in red, farm B in blue, and farm C in green).

Study II – PCV-3 detection in animals from farms without reproductive disorders

Figure 4.3. Phylogenetic NJ tree of aa of CAP gene constructed using Jones–Taylor–Thornton's model at 1000 bootstrap (the width of the branches is proportional to bootstrap P-values) and with GenBank reference samples included in Franzo et al., 2020a. The 39 PCV-3 ORF2 aa sequences were labeled with GenBank ID followed by farm identification, sow number, and sample number; F, for fetus samples; B, for brain; or L for lung tissue. Samples were colored by farm (farm A in red, farm B in blue, and farm C in green.

4.4 Discussion

The first description of PCV-3 infection was linked with reproductive losses with high viral loads in aborted fetuses (Palinski et al., 2017). Since then, this potential association has been reported in different countries. In China, a study found a higher frequency of the PCV-3 genome in sera from sows with a reproductive failure history (39/85, 45.9%) compared to healthy ones (23/105, 21.9%) (Zou et al., 2018). In a study from Brazil, PCV-3 viral DNA was found in pooled sera from sows delivering stillbirths, while it was absent in pool sera from sows with no stillbirth delivered (Tochetto et al., 2018). In one study from South Korea, the virus was suggested as the potential cause of an increased abortion and death rates of suckling piglets, since they could not demonstrate evidence of another pathogen in the tested animals (Kim et al., 2018a). A Hungarian newly populated farm experienced an increase of abortions and acute losses of neonatal piglets from the primiparous sows as well as an increase of stillborn and mummified fetuses compared to previous cycles; PCV-3 was the only pathogen found in these cases (Deim et al., 2020). However, the most unequivocal association to date of PCV-3 association with reproductive disease has been reported from the USA (Arruda et al., 2019). The authors found the presence of PCV-3 nucleic acids by means of in situ hybridization within the lesions of mummified and stillborn fetuses, especially from low-parity sows. Specifically, the PCV-3 genome was found in the smooth muscular cells of arteries of both the heart and kidney and in inflammatory cells in the heart.

However, considering that PCV-3 is a ubiquitous virus worldwide (Klaumann et al., 2018b), the mere detection of PCV-3 is not a clue to establish a potential association with disease. Therefore, in such scenarios, it is important to elucidate to which extent and in which frequency PCV-3 does circulate in normally performing farms. In fact, most of these studies reporting an association between PCV-3 and reproductive losses lack proper negative controls with standard reproductive parameters (Kim et al., 2018a; Arruda et al., 2019; Deim et al., 2020; Mora-Díaz et al., 2020). Therefore, the aim of the present study was to evaluate the frequency of PCV-3 in samples from farms with good reproductive parameters. It is noteworthy that the sampled farms had the reproductive parameters within the Catalan and Spanish averages regarding stillbirths (BDPork, Available at: http://bdporc.irta.es/informes/PartPublica/Datos%20publicos%20Anyo%202016.htm, accessed on 11 March 2020). The fact that the farms had good reproductive parameters did

not completely exclude the possibility of unnoticed reproductive problems in some sows (apparently primiparous ones) that may have caused few stillbirth and mummifications.
In the present study, PCV-3 DNA was only detected in sera from primiparous sows, mainly close to farrowing time. These results are in accordance with a study from Thailand that showed a higher viral load of PCV-3 in sera from low-parity sows when compared to older parity ones (Kedkovid et al., 2018b). These results were further confirmed by another study from the same research group, where 71% of the analyzed primiparous sows were positive to PCV-3 through the colostrum, while multiparous dams showed a shedding frequency of 33–43% (Kedkovid et al., 2018a). Interestingly, in the last study, the viremic and non-viremic sows did not show a significant different rate of total born or born-alive piglets (Kedkovid et al., 2018a), which is also in accordance with the present work where despite the presence of the virus in the herd, no reproductive losses were observed. Specifically, the mean of total piglets born per litter in farms A, B, and C were 14.39, 14.15, and 14.79, respectively.

A wide range of PCV-3 loads was found in positive fetuses in the current work, being the highest loads similar to the ones found in studies in which the presence of the virus in fetuses was attributed to reproductive losses, as well as associated to lesions (Fraile et al., 2009; Palinski et al., 2017; Arruda et al., 2019). In the present study, primiparous sows had a significantly higher number of PCV-3-infected fetuses with higher PCV-3 loads compared to those coming from multiparous sows. These findings may suggest that multiparous sows may have previously developed immunity that is able to prevent PCV-3 infection in their litters. In contrast, it is hypothesized that primiparous sows started gestation immunologically naïve against PCV-3, and the potential lack of immunity to the virus may have favored viral circulation in the herd and eventual transplacental transmission (Faccini et al., 2017). This situation would resemble the one observed in PCV-2, as piglets from primiparous sows are usually more susceptible to PCV-2 and PRRSV co-infection than piglets from multiparous sows (Dias et al., 2013).

The significantly higher PCV-3 detection rate in mummified than in stillborn fetuses found in the present study is also in agreement with the results obtained by Dal Santo et al. (2020), where almost 97% of the tested mummies were PCR-positive to the virus in commercial farms from Brazil (Dal Santo et al., 2020). However, in this latter study, most of the infected mummies came from farms experiencing reproductive losses; moreover, the fetuses also had co-infection close to 93% with other pathogens such as Porcine parvovirus,

PCV-2, or Leptospira spp. The presence of co-infecting pathogens would also explain the reproductive losses (Dal Santo et al., 2020), thus compromising the putative association with PCV-3 in the absence of alternative diagnostic methods (Arruda et al., 2019). An Italian study from farms experiencing reproductive failure in different stages of pregnancy demonstrated a high viral load of PCV-3 in tissues from stillborn and from aborted fetuses, while the most common pathogens that can cause reproductive disease were absent (Fraile et al., 2009). Similarly, mirroring with PCV-2, it is known that vertical transmission can happen at various stages of pregnancy, being able to cause reproduction losses as well as asymptomatic outcomes, depending on the timing of the virus infection and the degree of viral replication (Madson et al., 2011). Based on obtained results here, it may happen that PCV-3 infection mainly occurred earlier in the gestation, which would explain the higher percentage of infected mummified fetuses in comparison to stillborn.

Therefore, it is important to use additional diagnostic methods such as ISH in order to confirm the involvement of PCV-3 in the fetal lesions. Arruda et al. (2019) found messenger RNA matching with histological findings of multisystemic inflammation in several different tissues, including lung and brain. These features suggest that the virus might be replicating in these tissues, thus being the most probable cause of the disease. On the contrary, Faccini et al. (2017) observed that lungs from PCV-3 PCR-positive aborted fetuses did not show histological lesions despite having found high amounts of this virus in pools of tissues. Unfortunately, due the lack of available fixed tissue in the present study, histopathological evaluation and ISH were not able to be performed.

The phylogenetic analysis of the herein obtained sequences showed an extremely high percentage of nucleotide identity, as also observed in many other studies (Fux et al., 2018; Klaumann et al., 2018c; Arruda et al., 2019; Qi et al., 2019; Franzo et al., 2020a). The nucleotide identity was slightly higher when analyzing the virus found in fetuses from the same litter as well as from the respective sows, which further suggest the vertical transmission of the virus.

Through the phylogenetic analysis of the complete genome and the translated aa sequence from the ORF2 gene, it was possible to classify the 39 sequences recovered herein as PCV-3a (Franzo et al., 2020a). All 39 samples clustered together in a different branch from the available reference sequences when analyzed either the complete genome or the cap region (ORF2) tree, showing a highly similarity between them. However, when the translated Cap sequence was evaluated, some samples clustered separately, suggesting that

some of the mutations found within this region were non-synonymous, leading to different changes in the aa tree.

The present study demonstrated the presence of the PCV-3 genome in mummies and stillborn fetuses, supporting PCV-3's ability to cause intrauterine infections, even in farms with standard reproductive parameters. Moreover, a higher frequency of infection was found in primiparous sows and in mummified fetuses compared to multiparous dams and stillborn piglets, respectively. This study will help establish the 'infection background' of PCV-3 in standard farms without overt reproductive disorders. Although these results reinforce the vertical transmission of PCV-3, it is already too early to speculate about the importance of these findings, and further investigations are needed to ascertain the pathogenesis of PCV-3 infection.

CHAPTER 5

STUDY III

Porcine circovirus 3 detection in aborted fetuses and stillborn piglets from swine reproductive failure cases

Viruses. 2021, 13:264.
DOI: 10.3390/v13020264

Study III – PCV-3 detection in fetuses from reproductive failure cases

5.1 Introduction

Reproductive failure in sows represents an important drawback for the pig production sector, causing great economical losses worldwide (Christianson et al., 1992; Nathues et al., 2017; Althouse et al., 2019). When reproductive diseases affect dams during late-term gestation, the failure is manifested as abortions, stillborn and/or weak-born piglets or premature farrowing. Several different viruses are associated with reproductive problems, such as the PRRSV, PPV1, PCV-2, ADV, IAV, encephalomyocarditis virus (EMCV), and porcine enteroviruses (PEV), among others (Althouse et al., 2019). Lately, porcine circovirus 3 (PCV-3) has also been proposed as a pathogen associated with reproductive disease causation (Phan et al., 2016; Faccini et al., 2017; Palinski et al., 2017; Kim et al., 2018a; Tochetto et al., 2018, Zou et al., 2018; Arruda et al., 2019; Dal Santo et al., 2020).

PCV-3 was first described in 2015 and, since then, its role in different diseases has been debated. Although the viral DNA has been found in healthy pigs (Stadejek et al., 2017; Klaumann et al., 2018a) as well as in animals displaying various clinical conditions (Zhai et al., 2017; Kedkovid et al., 2018b; Shen et al., 2018; Qi et al., 2019), some recent reports have shown PCV-3 is most frequently associated with reproductive problems (Phan et al., 2016; Faccini et al., 2017; Palinski et al., 2017; Kim et al., 2018a; Tochetto et al., 2018, Zou et al., 2018; Arruda et al., 2019; Dal Santo et al., 2020). However, the mere detection of an enzootic virus in animals with clinical conditions is not sufficient to demonstrate its disease causality (Arruda et al., 2019). Despite the worldwide ubiquitous distribution of PCV-3 (Klaumann et al., 2018a; Study II), there is little evidence of PCV-3 detection within lesions of diseased animals (Arruda et al., 2019; Mora-Díaz et al., 2020). In consequence, it is of great importance to determine the frequency of the virus in farms with reproductive problems and assess its potential causality in these problems.

Thus, the present study aimed to investigate the frequency of PCV-3 detection in fetal/stillborn tissues of reproductive cases from Spanish farms. In addition, histopathologic description of positive cases and in situ detection of the virus was performed.

5.2. Material and methods

5.2.1. Sample selection

Tissue samples from 53 cases of reproductive failure submitted to the Veterinary Diagnostic Laboratory (DIAGNOS) of HIPRA in Spain (Amer, Spain) (n=51), and to the *Servei de Diagnòstic de Patologia Veterinària* de la *Facultat de Veterinària* de la *Universitat Autònoma de Barcelona* (UAB, Bellaterra, Spain) (n=2) between 2019 and 2020 were included in this study. The number of aborted/stillborn fetuses investigated per case ranged from 1 to 13. Cases originated from different conventional farms located in 15 different Spanish provinces.

From each fetus/stillborn piglet, fresh (heart, lung, spleen, kidney, thymus, and liver) and fixed tissues (heart, lung, spleen, kidney, thymus, liver, intestine, and in some cases cerebrum and cerebellum) were collected during the necropsy. Fresh tissues were pooled (per case) and homogenized (10% w/v in PBS), while the formalin-fixed tissues were handled for histopathologic evaluation. The crown-rump length (CRL) of the specimens (aborted fetus or stillborn) was measured to estimate the pregnancy timing.

5.2.2. DNA extraction, PCV-2, and PCV-3 qPCR

DNA extraction was done from 200 µL of supernatant from the pooled macerated tissues (heart, lung, spleen, kidney, thymus, and liver) using MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®, Foster City, CA, USA) following the manufacturer's protocol.

A real-time quantitative PCR (qPCR) analysis was performed targeting PCV-3 as previously described (Franzo et al., 2018b; Study I). The qPCR results were expressed in log10 of PCV-3 DNA copies/mL of supernatant of macerated tissue samples; the limit of detection of the technique is 10^3 copies of DNA/mL (Franzo et al., 2018b; Study I).

PCV-2 qPCR was performed using LSI VetMAX™ Porcine PCV2 Quant Kit (Applied Biosystems®, Foster City, CA, USA), according to the manufacturer's protocol. The qPCR results were expressed in log10 of PCV-2 DNA copies/mL of supernatant of macerated tissue samples; the limit of detection of the technique is 4 log10 copies of DNA/mL as indicated by the manufacturer.

5.2.3. PRRSV and PPV1 detection

RNA extraction was done from 100 µL of supernatant from the same pooled macerated tissues as described before using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The PPRSV real-time PCR analysis was performed using a previously described technique (Martínez et al., 2008).

Two hundred microliters of the same pooled macerated tissues were used to perform DNA extraction by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. PPV1 presence was assessed using real-time PCR analysis adapted from (Miao et al., 2009).

5.2.4. Histopathology, PCV-2 immunohistochemistry, and PCV-3 *in situ* hybridization

Available tissues from each case (heart, lung, spleen, kidney, thymus, liver, intestine, cerebrum, and/or cerebellum) were fixed in 10% neutral buffered formalin. Fixed samples were dehydrated, embedded in paraffin wax, sectioned at 4 µm, and stained with hematoxylin and eosin (HE). Subsequent paraffin block cuts were used to assess PCV-2 and PCV-3 by IHC and ISH, respectively, on selected samples.

PCV-2 qPCR positive samples were tested by PCV-2 IHC (Grau-Roma et al., 2009). PCV-3 qPCR positive samples with Ct ≤ 30 were analyzed by a PCV-3 ISH using the RNAscope® 2.5 HD Reagent Kit-RED (Advanced Cell Diagnostics, Newark, CA, USA) according to the manufacturer's instructions (Wang et al., 2012). Paraffin-embedded tissues from the selected cases were mounted on Dako Flex® slides (Ref. K8020). The slide sections were dried overnight in an incubator at 37 °C. Slides were deparaffinized by being immersed two times for 5 min in xylene followed by two times for 3 min in 100% alcohol and then air dried for 10 min at RT. The pre-treatment was made with RNAscope® Hydrogen Peroxide at RT for 10 min, rinsed with distilled water, and then boiled for 20 min at 99 °C in the RNAscope® 1X Target Retrieval Reagent. Subsequently, slides were rinsed in distilled water, and washed in fresh 100% alcohol for 1 min. After air drying, a hydrophobic barrier was drawn around each tissue section using the ImmEdge™ pen. Afterwards, the RNAscope® Protease Plus was applied and incubated for 10 min at 40 °C in the HybEZ™ oven and washed with distilled water. The PCV-3 Rep target probe (catalogue No. 491021) as well as the negative DapB probe (catalogue No. 310043) were pre-heated for 10 min at 40 °C and then hybridized for 2 h at 40 °C in the HybEZ™ Oven. Slides were washed with RNAscope® 1X Wash Buffer and followed by six amplification steps (RNAscope ® 2.5

AMP 1–6) interspersed with washes of RNAscope® 1X Wash Buffer. Slides were incubated with red chromogenic detection solution for 10 min at RT, followed by a counterstain with 50% hematoxylin, and rinsed with tap water for 10 min. After air drying, slides were quickly mounted by submerging them into fresh pure xylene and using EcoMount (Biocare Medical, Pacheco, CA, USA) to coverslip them. All tested slides (one or two) per case were also assayed with a negative control probe, as recommended by the manufacturer's protocols. A positive control with the PCV-3 Rep probe was also used for each ISH batch performed. The amount of cells stained by the ISH was scored semi quantitatively with "–" for no infected cells, "+" for low amount (<10 labelled cells as a mean of 5 fields examined at x200 magnification), "++" for medium amount (10–50 la-belled cells), and "+++" for a high amount of cells (>50 labelled cells) per tissue sample containing the PCV-3 genome.

5.2.5. PCV-3 and PCV-2 sequencing and PCV-3 phylogenetic analysis

All qPCR PCV-2 positive samples were selected for sequencing the ORF2 to establish the potential genotype involved using primers described by Oliver–Ferrando et al. (2016), with slight modifications (Study I).

Positive PCV-3 qPCR samples that showed a Ct ≤ 30 were selected for complete genome sequencing. PCV-3 DNA was amplified using a previously described panel of primers (Fux et al., 2018), purified with ExoSAP-IT® Express PCR product Cleanup Kit (Applied Bi-osystems®, Foster City, CA, USA) according to the manufacturer's protocol, and se-quenced by the Sanger method (ABI3730XL-Macrogen Europe, Madrid, Spain). The quality of the sequences was analyzed by Finch TV software and trimmed in BioEdit v. 7.2.6 (Hall, 1999). To achieve the PCV-3 complete genome, the amplicons were assembled using the online version of MAFFT version 7 (Katoh et al., 2019) and aligned with a set of published reference samples selected by Franzo et al. (2020a) (available at: https://www.mdpi.com/1999-4915/12/3/265/s1, accessed on 15 October 2020) using ClustalW available in BioEdit vs. 7.2.6 (Hall, 1999). PCV-3 ORF2 gene and the translated ORF2 amino acid (aa) region were also aligned with the same selected references. For the phylogenetic analysis, the best substitution method was chosen based on the lowest Bayesian Infor-mation Criterion (BIC) score calculated using MEGA X software (Kumar et al., 2018), either for the com-plete genome analysis as for the ORF2 or the ORF2 aa analysis. The maximum likelihood phylogenetic tree of the complete genome was constructed using the Tamura–Nei model plus Gamma and I (G+I) distribution, while the ORF2 analysis was

performed using Hasegawa–Kishino–Yano (HKY) model (Hasegawa et al., 1985) plus Gamma distribution, and the translated ORF2 region was analyzed using Jones–Taylor–Thornton's model (Jones et al., 1992) plus Gamma distribution. All trees were built with 1000 bootstrap replicates using MEGAX software (Kumar et al., 2018). The nucleotide (nt) and aa identity matrices among sequences were obtained using Clustal Omega (Sievers and Higgins, 2014). The PCV-3 sequences obtained in this study are available at the NCBI GenBank with the accession numbers MW167063–MW167068.

5.3. Results

5.3.1. PCV-3 detection by qPCR

From the 53 collected cases of reproductive failure, 18 (33.9%) were PCV-3 qPCR positive. From these 18 samples, 8 showed quantifiable viral loads (from 3.3 to 8.7 log10 copies/mL of supernatant of macerated tissue samples). Among these 8 samples, 6 had high viral loads with Ct levels ≤30 (from 5.9 to 8.7 log10 copies/mL).

PCV-2 DNA was detected in 5 out of the 53 cases (9.4%). These 5 positive cases had low viral loads, below the BLQ. PRRSV RNA was found in 4 out of the 53 cases (7.5%), and PPV1 was not detected. Only two out of the 53 (3.8%) cases displayed coinfection (Table 5.1): one case was positive for PCV-3 and PRRSV, and the other one to PCV-3, PCV-2, and PRRSV. Overall virological results are summarized in Table 5.2.

The CRL of the fetus/stillborn piglets ranged from 6 to 32 cm. Most of the fetal death occurred in the last third of gestation.

Table 5.1. Frequency and range of fetal/stillborn piglet size of qPCR positive cases by pathogen and coinfections.

Agent	Positive (Out of 53)	%	CRL (cm) Range
PCV-3	16	30.2	15–32
PCV-2	4	7.5	16–30
PRRSV	2	3.8	20–28
PCV-3/PRRSV	1	1.9	23–27
PCV-3/PCV-2/PRRSV	1	1.9	6–22 *

CRL: Crown-to-rump, * The highly variable range was due to the presence of mummified fetuses among the aborted ones.

Table 5.2. Number of fetuses or stillborn piglets, range of crown-to-rump length (CRL), and RT-qPCR or qPCR results obtained in those cases positive at least for one virus.

Case No.	Number of fetuses	CRL (cm) range	PCV-3 Ct values (log10 copies/mL)	PCV-2 Ct values (log10 copies/mL)	PRRSV Ct values
2	5	22-30	Neg	37.0 (BLQ)	Neg
3	1	22	24.4 (6.5)	Neg	Neg
4	2	22-28	35.6 (BLQ)	Neg	Neg
6	1	23	Neg	Neg	32.9
9	1	30	37.7 (BLQ)	Neg	Neg
13	3	23	37.5 (BLQ)	Neg	Neg
14	13	20-28	Neg	Neg	24.91
17	8	26-32	37.4 (BLQ)	Neg	Neg
22	2	16-21	Neg	35.5 (BLQ)	Neg
24	5	23	38.2 (BLQ)	Neg	Neg
30	2	22-25	Neg	38.4 (BLQ)	Neg
32	6	25-28	37.4 (BLQ)	Neg	Neg
35	5	15-20	24.3 (7.7)	Neg	Neg
36	4	6-22	24.3 (6.5)	37.2 (BLQ)	32.7
39	11	15-25	26.5 (5.9)	Neg	Neg
40	3	16-23	18.0 (8.5)	Neg	Neg
41	2	22-25	Neg	38.3 (BLQ)	Neg
42	10	23-27	33.8 (3.6)	Neg	27.1
43	2	22	35.5 (BLQ)	Neg	Neg
44	10	17-23	36.5 (BLQ)	Neg	Neg
47	6	23-27	34.7 (3.30)	Neg	Neg
48	2	23	37.9 (BLQ)	Neg	Neg
49	2	15	37.8 (BLQ)	Neg	Neg
52	6	27-28	16.9 (8.71)	Neg	Neg

BLQ: Below the quantification limit

5.2.2. Histologic evaluation, PCV-2 IHC, and PCV-3 ISH

In most of the cases, autolysis and no histologic lesions were observed. Few cases (6 out of 53) had histologic lesions; three animals had mild multifocal interstitial pneumonia (case Nos. 5, 22, and 52) and three had systemic lymphoplasmacytic periarteritis (case Nos. 3, 40 and 52).

Four out of five PCV-2 qPCR positive samples did not show histological lesions (only case No. 22 showed interstitial pneumonia) and were negative by PCV-2 IHC. No histological lesions were observed in the four PRRSV qPCR positive cases.

PCV-3 was detected by ISH in 4 out of the 6 selected samples with high viral loads (PCV-3 qPCR Ct ≤ 30) (Table 5.3). These four PCV-3 ISH positive cases (case Nos. 3, 35, 40, and 52) had no evidence of coinfections. Two cases, Nos. 36 and 39, with qPCR Ct < 30 but negative by ISH, had marked signs of autolysis in tissues.

No viruses were found in the case with interstitial pneumonia (case No. 5).

The PCV-3 genome was mainly found in the smooth muscle cells of arteries in different tissues, and also in macrophage-like cells in lung and kidney (Figure 5.1 a-h). Fetuses from three out of the four ISH-positive cases showed mild inflammation (mainly mild lymphocytic infiltration (LI)) in the same area of PCV-3 ISH labeling (Nos. 3, 40, and 52). The most abundant labeled cells were found in case Nos. 40 and 52. Case No. 40 showed almost all the analyzed tissues positive to PCV-3 by ISH; however, only the heart tissue showed evident histopathologic lesions. In case No. 52, PCV-3 was detected in all tested tissues, and arteritis and perivascular inflammation were observed in most of them (Figure 5.1 c–f), except in cerebrum and cerebellum (Figure 5.1 g,h). Tissues such as the heart and spleen of case No. 3 and the lung of case No. 35 contained low amounts of labeled cells without any histological lesions.

5.3.3. Sequencing and phylogenetic analysis

None of the PCV-2 positive samples were successfully sequenced due the low amount of virus in the investigated samples; therefore, PCV-2 genotyping was unable to be assessed.

Table 5.3. Crown-to-rump (CRL) range of the 6 PCV-3 qPCR positive samples with high viral loads by case no., results of PCV-3 tests (qPCR and ISH), and histologic lesions.

Case No.	CRL (cm) Range	Ct Values (log10 Copies/mL)	ISH Results		Histological Lesions
3	22	24.4 (6.5)	Heart	+	Heart: No lesions
			Lung	++	Lung: mild LI in arteries
			Intestine	+	Intestine: mild perivascular LI
			Spleen	+	Spleen: No lesions
			Kidney	+	Kidney: mild LI in pelvis
			Liver	–	Liver: No lesions
35	15–20	24.3 (7.7)	Heart	–	Heart: No lesions
			Lung	+	Lung: tunica media swelling in lung arteries' wall
			Kidney	–	Kidney: No lesions
			Liver	–	Liver: No lesions
36	6–22	24.3 (6.5)	Heart	–	Heart: Autolysis
			Kidney	–	Kidney: Autolysis
			Liver	–	Liver: Autolysis
39	15–25	26.5 (5.9)	Heart	–	Heart: Autolysis
			Lung	–	Lung: Autolysis
			Intestine	–	Intestine: Autolysis
			Spleen	–	Spleen: Autolysis
			Kidney	–	Kidney: Autolysis
			Liver	–	Liver: Autolysis
			Umbilical cord	–	Umbilical cord: Autolysis
40	16–23	18.0 (8.5)	Heart	+++	Heart: mild LI in smooth muscles of arteries, mild lymphoplasmacytic endocarditis and myocarditis
			Lung	++	Lung: No lesions
			Intestine	+	Intestine: No lesions
			Kidney	+	Kidney: No lesions
			Liver	++	Liver: Autolysis
			Thymus	–	Thymus: No lesions
52	27–28	16.9 (8.71)	Heart	++	Heart: mild LI in perivascular connective tissue
			Lung	++	Lung: hemorrhage, IP' and mild LI in arteries
			Spleen	++	Spleen: hemorrhage, mild LI' and vacuolization in smooth muscles of arteries' wall
			Kidney	++	Kidney: mild LI in smooth muscles of arteries
			Liver	++	Liver: mild LI in arteries and hepatocytes with vacuolization
			Cerebrum	+++	Neuropil vacuolization
			Cerebellum	++	Multifocal cortical hemorrhages

–: non-infected cell; +: low amount of PCV-3 positive cells; ++: medium amount of PCV-3 positive cells; +++: high amount of PCV-3 positive cells. LI: lymphocytic infiltration; IP: interstitial pneumonia

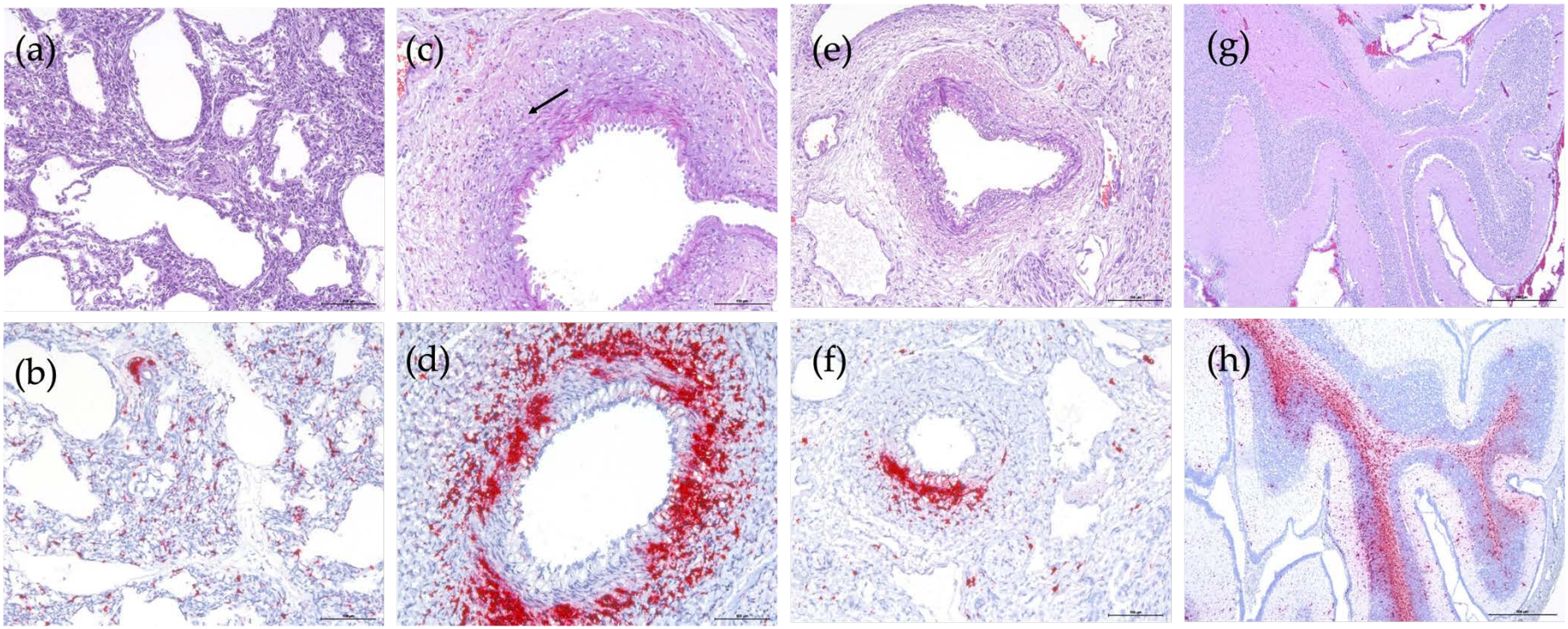

Figure 5.1. Histology (H&E stain, a,c,e,g) and PCV-3 ISH (hematoxylin counterstain, b,d,f,h) results. (a) Fetal lung from case No. 3 with no apparent histopathologic lesions. (b) PCV-3 genome in macrophage-like cells and in a perivascular area in lung fetus from case No. 3. (c) Moderate tunica media swelling in a splenic artery with a mild lymphocytic in-filtration at perivascular and arteriolar locations (arrow) of a stillborn piglet from case No. 52. (d) PCV-3 detection in smooth muscle of spleen artery and perivascular inflammation in the same fetus. (e) Kidney–pelvis artery of a stillborn piglet from case No. 52. (f) PCV-3 detection in smooth muscle and in lamina propria of a kidney artery of the same fetus. (g) Cerebellum from a stillborn piglet with no apparent histopathologic lesions of fetus from case No. 52. (h) High amount of PCV-3 nucleic acid in cerebellum white matter and mild-to-moderate in grey matter of the same animal

In the six samples with high PCV-3 viral loads (Ct ≤ 30), sequencing was successfully performed. Thus, the nucleotide identity of the PCV-3 complete genome of the analyzed samples (n=6) demonstrated that five out the six showed high identity between each other (Table 5.4). However, one of the sequences (case No. 40) showed lower identity (96.7% to 97.8%) when compared to the other five obtained herein and also when compared to the set of reference sequences proposed by Franzo et al. (2020) (Table 5.4). Thus, this sequence slightly exceeded the maximum within-genotype genetic distance of 3% at the complete genome analysis in order to be classified as PCV-3a, as previously proposed (Franzo et al., 2020a).

Table 5.4. Range in percentage (%) of complete genome of nt, ORF2 nt, and ORF2 aa identity between PCV-3 sequences retrieved in this study and selected reference sequences (Franzo et al. 2020a).

Sequences from Case No.	**Sequences from Case Nos. 3, 35, 36, 39, 52 (%)**			**Set of Published Reference Samples Selected by Franzo et al. (2020a) (%)**		
	Complete Genome	**ORF2**	**ORF2 aa**	**Complete Genome**	**ORF2**	**ORF2 aa**
3, 35, 36, 39, 52	99.2–100	98.9–100	98.6–100	98.4–100	97.2–100	95.3–100
40	97.5–97.7	95.9–96.1	96.7–97.2	96.7–97.8	95.4–96.6	94.9–98.1

The phylogenetic analysis of the whole PCV-3 genome displayed two main clusters (Figure 5.2). Five out of the six sequences analyzed in this study belonged to the same cluster, together with the reference sequences previously genotyped as PCV-3a (Franzo et al. 2020a). The sample from case No. 40 showed the highest number of single nucleotide polymorphisms (SNP) found throughout the complete genome (Table 5.5) when compared to the other cases and located in a separated branch in the phylogenetic tree (Figure 5.2). Similar results were found when ORF2 sequences were phylogenetically analyzed, as case No. 40 also clustered with samples classified as PCV-3a but in a separated branch of the phylogenetic tree (Figure 5.3). When the number of SNPs was compared throughout the ORF regions, ORF2 was the one containing the highest number of SNPs (9–26 SNPs in the cap gene, while there were 1–8 SNPs in the rep gene).

Study III – PCV-3 detection in fetuses from reproductive failure cases

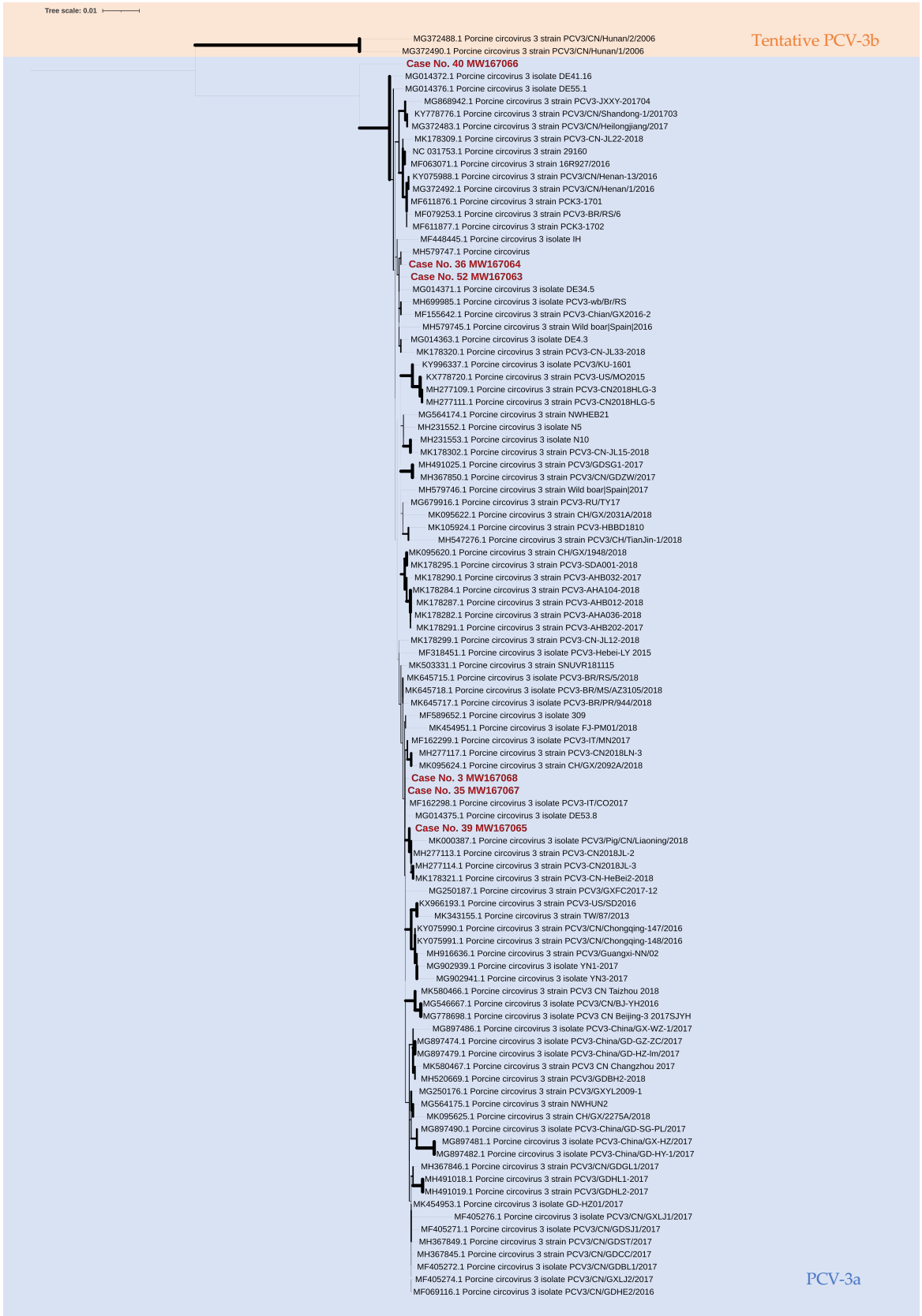

Figure 5.2. Phylogenetic analysis of PCV-3 complete genome sequences from this study, and the PCV-3 reference sequences from Franzo et al. (2020a). The maximum likelihood tree was constructed with the Tamura–Nei model with Gamma plus I distribution (1000 replicates). The width of the branches is proportional to bootstrap values, and the scale bar indicates nucleotide substitutions per site. Sequences obtained in the present study are colored in red; sequences classified as PCV-3a are in the blue shadow, and the sequences of a tentative PCV-3b in orange shadow.

Study III – PCV-3 detection in fetuses from reproductive failure cases

Figure 5.3. Phylogenetic analysis of PCV-3 ORF2 nt sequences from this study, and the PCV-3 reference sequences from Franzo et al. (2020a). The maximum likelihood tree was constructed with the Hasegawa–Kishino–Yano model with Gamma distribution (lowest BIC score) at 1000 replicates. The width of the branches is proportional to the bootstrap values, and the scale bar indicates nucleotide substitutions per site. The six sequences obtained herein are colored in red; sequences classified as PCV-3a are in the blue shadow and the sequences of a tentative PCV-3b in orange shadow.

Study III – PCV-3 detection in fetuses from reproductive failure cases

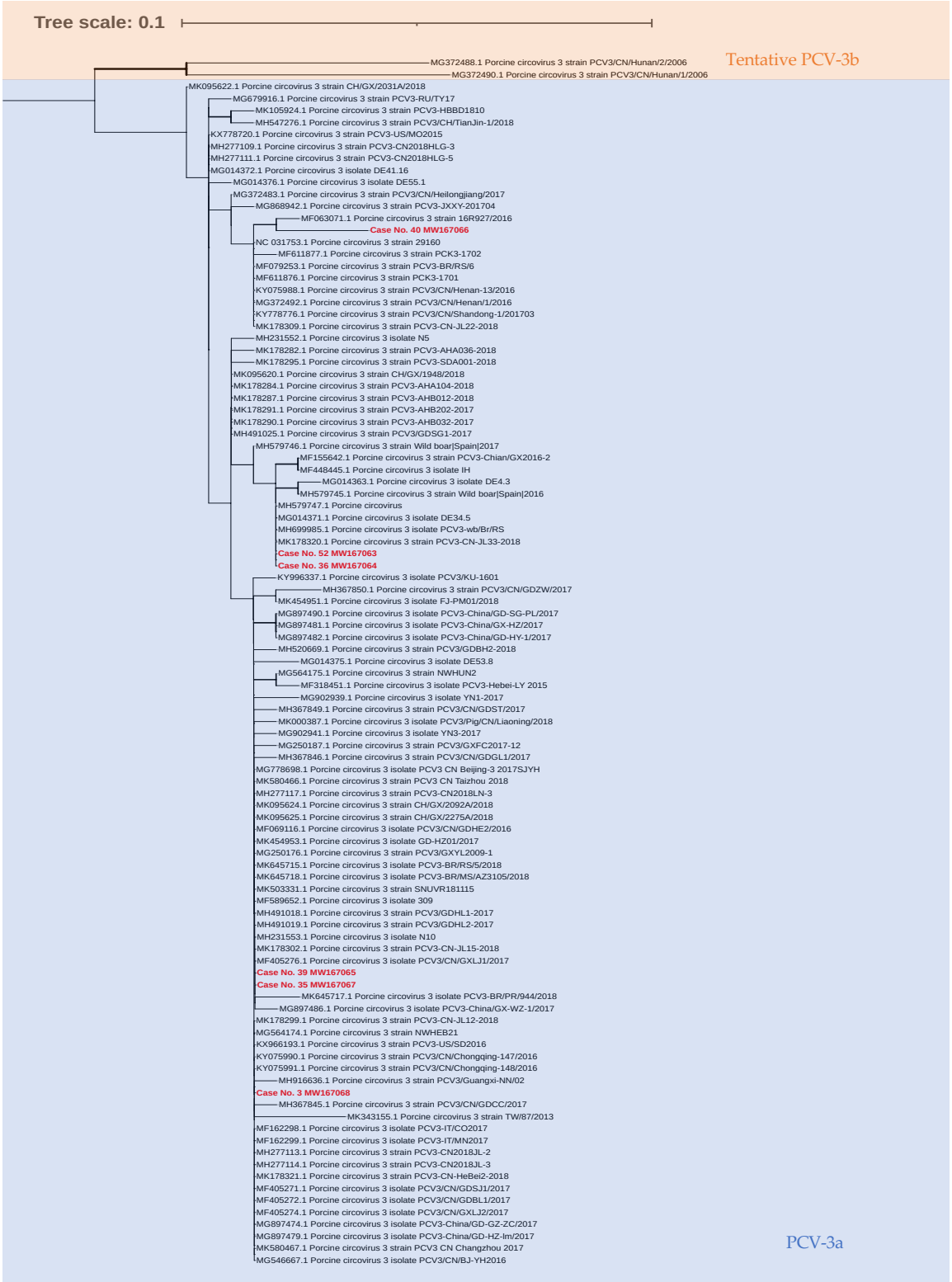

Figure 5.4. PCV-3 phylogenetic analysis tree of aa of CAP protein from sequences from this study, and the PCV-3 references sequences from Franzo et al. (2020a). The maximum likelihood tree was constructed with Jones–Taylor–Thornton's model with Gamma distribution (lowest BIC score) at 1000 replicates. The width of the branches is proportional to bootstrap values, and the scale bar indicates nucleotide substitutions per site. The six sequences obtained herein are colored in red; sequences classified as PCV-3a are in the blue shadow and the sequences of a tentative PCV-3b in orange shadow.

Study III – PCV-3 detection in fetuses from reproductive failure cases

Table 5.5. Table of SNPs of sequences retrieved in this study compared to the PCV-3 reference of NCBI (NC031753). The genomic positions are displayed in 5'-3'except for the non-synonymous SNPs from the cap gene (displayed 3'-5').

Region	UTR							REP													UTR									
Genomic position (NC031753)	36	40	54	76	79	115	178	317	515	557	579	662	725	800	837	876	890	968	1007	1061	1136	1148	1149	1160	1174	1182	1183	1328	1329	1333
Nt	G	G	A	C	T	G	T	A	C	A	T	G	A	T	T	C	C	T	A	G	G	G	T	A	G	G	C	A	T	C
Case 3											G		T		C							T		T						
											S122A																			
Case 35											G		T		C									T						
											S122A																			
Case 36															C								A	T			A			
Case 39							C			T	G	T	T		C									T						T
											S122A																			
Case 40		A	T	T	A	T		G	A					A	C	T		A	C	T	T			T	A	A	G	C	C	
Case 52	C				A										C		T							T						

Region	CAP																																				
Genomic position (NC031753)	1345	1384	1405	1426	1438	1482	1483	1519	1533	1579	1583	1585	1600	1670	1693	1696	1714	1726	1732	1751	1756	1758	1795	1819	1825	1834	1846	1900	1901	1902	1910	1933	1939	1941	1951	1968
Nt	G	G	C	C	T	A	G	A	G	A	T	A	C	A	C	A	T	T	T	G	T	C	G	A	T	T	T	T	C	G	G	T	C	T	T	C
Case 3									T			C						C		C	C					A	G		T	T	A					
									L150I											T77S										R27K		A24V					
Case 35									T									C		C	C					A	G		T	T	A					
									L150I											T77S										R27K		A24V					
Case 36									T				T	T				C		C				T		A	G			T		G				
									L150I					F104Y						T77S																	
Case 39									T									C		C	C					A	G		T	T	A					
									L150I											T77S										R27K		A24V					
Case 40	T	A	T	T	C	T	A	T		T	C					T	G	C	A	C		A		C	C	A	G	C		T		T	G	C	G
						S167T					K133R									T77S		A75S														A5P
Case 52									T					T	T			C		C						A	G			T		G				
									L150I					F104Y						T77S																	

Moreover, the cap region was translated and the aa sequences from the inferred ORF2 protein were phylogenetically analyzed (Figure 5.4). All of them were grouped in the same cluster including case 40, in agreement with the low number of SNPs (26 SNPs) found that corresponded to five non-synonymous mutations in this sequence (Table 5.5).

5.4. Discussion

In the present study on reproductive failure cases, PCV-3 was the viral agent detected at a higher frequency, whereas PPV1 was not found, and PCV-2 and PRRSV were detected in less than 10% of these cases. Notably, the global rate of infection by viruses of the present study is fairly high (around 45%). Although it cannot be proven that the pathogens found herein were the cause of the reproductive losses in the studied farms, the results found in the present work fit well with the fact that an average of more than 50% of reproductive failure cases are not of infectious origin (Christianson et al., 1992). There are also a significant number of bacterial pathogens that may account for reproductive disorders (Althouse et al., 2019). The three viruses studied here, apart from PCV-3, are already well recognized as putative causative agents of reproductive disease (Hermann et al., 2005; Brunborg et al., 2007; Madson et al., 2009; Truyen et al., 2019), and the results obtained in the present studies reinforce (Arruda et al., 2019; Mora-Díaz et al., 2020; Temeeyasen et al., 2021) the potential of PCV-3 as a pathogen able to cause reproductive problems, as suggested elsewhere (Arruda et al., 2019; Mora-Díaz et al., 2020; Temeeyasen et al., 2021). The present study did not aim at establishing potential bacteria causing reproductive disorders; however, no lesions compatible with bacterial infections were observed during histologic evaluation in the studied tissues of fetuses and stillborn piglets.

From all tested cases, only five were positive to PCV-2 by qPCR. These PCV-2 positive cases had very low viral load in tissues, did not present any typical pathologic lesion associated to this infection, such as myocarditis (Segalés, 2012), and were negative by IHC. Altogether, results suggest that the reproductive problems detected in these cases were probably not associated with this virus. Since PRRSV was the third pathogen detected most frequently in the samples assessed, it cannot be ruled out that this virus was in-volved in the reproductive problems observed in affected farms. However, no evidence of significant histologic lesions was observed in tissues from fetuses infected by PRRSV. A similar study performed in 2005, in which the presence of different viruses (PRRSV, ADV, PPV1 and PCV-2) in reproductive failures was investigated, concluded that PRRSV was the main

pathogen responsible for late-term abortion in Spain as this virus was detected in 9 out of 100 reproductive failures (Maldonado et al., 2005). Therefore, the results obtained in both Spanish studies demonstrate that the frequency of PRRSV detection in reproductive failure cases in Spanish herds is similar fifteen years later. Maldonado et al. (2005) also found a low frequency of PCV-2 DNA (1/100) by PCR and no positive cases by ISH. The lack of PPV1 detection in the studied cases also coincides with the abovementioned study and could be explained by the wide use of vaccination against this agent (Maldonado et al., 2005). However, the comparison of PCV-3 frequency with the study of Maldonado et al. (2005) is not possible due the fact that this virus was not described at that time, even though it is known through retrospective studies that PCV-3 has been circulating in Europe since the mid-1990s at least (Klaumann et al., 2018a). Thus, if PCV-3 would have been investigated at that time, we hypothesize that it would probably have been found.

PCV-3 DNA has been widely detected in samples from healthy animals (Stadejek et al., 2017; Franzo et al., 2018a; Klaumann et al., 2018a; Zou et al., 2018; Ye et al., 2018) as well as in mummified fetuses and stillborn piglets from farms without reproductive problems (Study II). The simple assessment of viral DNA in cases of reproductive problems also has been widely performed (Faccini et al., 2017; Tochetto et al., 2018; Zou et al., 2018; Deim et al., 2019; Dal San et al., 2020), but the detection of a viral genome alone does not imply the causality of a disease. Therefore, the next step was to investigate the presence of PCV-3 within lesions in fetuses/stillborn piglets in farms displaying re-productive problems, as suggested (Arruda et al., 2019). Two out of the six cases with high loads of PCV-3 in tissues showed a lack of histological lesions. These results are not surprising since other studies have already mentioned the lack of lesions even in the presence of great amounts of PCV-3 DNA (Faccini et al., 2017; Temeeyasen et al., 2021). A Hungarian group found high loads of PCV-3 in tissues from aborted and weak-born piglets with no gross lesions (Deim et al., 2019). Indeed, this lack of gross lesions in aborted fetuses is not uncommon, thus microscopic lesions are a more valuable indicator of viral abortion (Althouse et al., 2019; Arruda et al., 2019). Additionally, Faccini et al. (2017), also found high PCV-3 loads (10^{10} genome copies/mL) in pools of lung tissues from fetuses and stillborn piglets, without detection of microscopic lesions. However, in the present study, fetuses from PCV-3 ISH positive cases (Nos. 3, 35, 40, and 52) had mild-to-moderate inflammation (mainly lymphoplasmacytic infiltration) in arteries in different tissues (case Nos. 3, 35, and 52), or mild myocarditis and endocarditis (case No. 40). These findings suggest a PCV-3 tropism

mainly for vasculature, like what has been described in piglets with multisystemic inflammation (Phan et al., 2016; Arruda et al., 2019; Mora-Díaz et al., 2020; Temeeyasen et al., 2021). Some of the animals from these latest reports also showed lesions compatible PDNS (Phan et al., 2016; Palinski et al., 2017; Arruda et al., 2019). Moreover, PCV-3 detection through ISH was also observed in a wasting pig in South Korea showing respiratory distress with perivascular and peribronchiolar lymphocytic infiltration (Kim et al., 2018a). This animal was also positive to other pathogens such as PRRSV, *Streptoccocus suis* and *Mycoplasma hyopneumoniae* (Kim et al., 2018a). Additionally, a recent experimental PCV-3 inoculation performed in the USA provided evidence that PCV-3 is able to produce multi-systemic inflammation as well as subclinical infections (Temeeyasen et al., 2021). Specifically, all animals of two different inoculated groups had prolonged viremia, but only 2/4 and 3/4 showed histopathologic lesions (Temeeyasen et al., 2021). Thus, Temeeyasen et al. (2021) suggested that the PCV-3 pathogenesis is complex and of multifactorial nature. The results presented herein, where most of the cases (4 out of 6) with high PCV-3 loads had histopathologic findings, support the abovementioned statement that the presence and also the severity of the lesions might be multifactorial (Arruda et al., 2019; Mora-Díaz et al., 2020; Temeeyasen et al., 2021). Moreover, Temeeyasen et al. (2021) speculated that the higher number of positive tissues by qPCR compared to positive ISH could be explained by viremia and was not due to in situ viral replication.

Franzo et al. (2020a) have proposed a single PCV-3 genotype (PCV-3a) after analyzing most of the PCV-3 available sequences in public databases. Three characteristics were suggested for the definition of PCV-3 genotypes: (1) the limit of the maximum within genotype raw genetic distance of 3% at complete genome analysis, (2) 6% at the ORF2 level, and (3) a 90% minimum of bootstrap. Thus, the phylogenetic analysis of the six PCV-3 sequences obtained in this study demonstrated that five out of the six sequences were unequivocally classified as PCV-3a by (Franzo et al., 2020a). All five sequences showed high nt identity between each other as well as when compared to the set of selected samples classified as PCV-3a by Franzo et al. (2020a). The five sequences also clustered together in all constructed phylogenetic trees. However, case No. 40 showed the nt identity of the complete genome sequence close to the limit of the genetic distance proposed to define the PCV-3 genotype (Franzo et al., 2020a). In fact, this sequence only strictly fulfilled one out of the three characteristics (the second one mentioned above) to classify PCV-3 sequences within the unique proposed genotype. Therefore, the sequence of case No. 40 was within the

limit of the newest proposed genotype. This finding reinforces the importance of the continuous surveillance of the sequences circulating in the field.

The present work demonstrated the presence of the PCV-3 genome within (mild-to-moderate) histological lesions of aborted fetuses. Thus, PCV-3 should be considered as a potential causative pathogen for reproductive failure. Based on the present findings, further studies are needed to elucidate the specific pathogenesis of PCV-3 infection of the pregnant sow, the frequency at which the virus can cause lesions and disease, as well as its economic impact on the sector.

CHAPTER 6

STUDY IV

Porcine circovirus 3 associated wasting in post-weaning pigs

Submitted for publication

Study IV - Porcine circovirus 3 associated wasting in post-weaning pigs

6.1 Introduction

Disease causality demonstration for a ubiquitous agent is not an easy task, and most of the reports published to date on PCV-3 (Opriessnig et al., 2020) are based on the detection of viral genome in sick animals. However, the mere presence of nucleic acid in a sample does not constitute evidence of disease causality (Arruda et al., 2019). Among all published studies, only few of them showed the presence of PCV-3 within lesions, which may account for this virus as a cause of the observed clinical condition (Phan et al., 2016; Arruda et al., 2019; Kim et al., 2018a). Moreover, only one has demonstrated a significant amount of PCV-3 nucleic acid in the damaged tissues of post-weaning pigs, further suggesting the role of this virus in causality (Arruda et al., 2019).

To date, there are no peer-reviewed published reports of PCV-3 associated disease in pigs from Europe, although cases with high viral loads in stillborn and lactating piglets from United Kingdom and Spain have been described (Williamson et al., 2019; Study III). Here, we report the first case of post-weaning disease associated with PCV-3 described in Europe.

6.2 Material and methods

Since January 2020, a Portuguese 200 sow-farm with a 3-week farrowing batch production system weaning 30.5 piglets per sow/year suffered from a clinical disorder in post-weaning piglets. It consisted of growth-retarded animals showing thrown-back ears (the condition has been vulgarly named as “aviator” or "flying pig syndrome"), wasting and rough hair (Figure 6.1). The initial incidence of the condition was 1.25%, increasing up to 3.85% in April 2020 and decreasing to 0.90% in November on the same year.

By October and November 2020, the *Servei de Diagnòstic de Patologia Veterinària* (SDPV) of the *Facultat de Veterinària* at the *Universitat Autònoma de Barcelona* received four affected animals and two non-affected ones to be used as negative controls. All animals were subjected to an exhaustive necropsy.

Tissue samples (lymph nodes, Peyer’s patches, tonsil, heart, central nervous system – cerebrum, cerebellum, and pons –, kidneys, lungs, spleen, large and small bowel, liver, and/or nasal turbinate) were collected and fixed by immersion in a 10% buffered formaldehyde solution for 48 hours. Tissues were subsequently embedded in paraffin wax, sectioned at 3 µm, and stained with HE for routine histology assessment.

The same tissues were used to assess the presence PCV-3 genetic material by ISH using RNAscope® technology (Study III). Also, PCV-2 and PRRSV antigens were investigated by IHC techniques (Rosell et al., 1999; Segalés et al., 2002).

Figure 6.1. Post-weaning pig suffering from the so-called "flying pig syndrome" with wasting and thrown-back ears.

6.3 Results

Macroscopic evaluation of clinically affected pigs showed poor body condition with rough hair but no evidence of gross lesions in internal organs. Non-diseased animals were of smaller size but were clinically healthy and did not show gross lesions as well.

Microscopically, diseased pigs had multi-organic, moderate to severe lymphoplasmacytic periarteritis and arteritis in mesenteric arteries (4/4), heart (4/4), kidneys (4/4), spleen (4/4), portal arterioles (3/4), meninges (2/4), lungs (2/4), and/or stomach (2/4). The histological features of affected arteries were circumferential or segmental lymphoplasmacytic inflammatory infiltration (Figure 6.2 a). These inflammatory infiltrates expanded and disrupted the periarteriolar connective tissue and tunica adventitia, only affecting the tunica media and intima in the most severe cases. Smooth muscle cells of the tunica media showed a mild-to-intense vacuolization with loss of cytoplasm borders definition. Occasionally, endothelial cells were plumped to the lumen and leukocytes attached to the endothelium displaying rolling and exocytosis. Besides the blood vessel lesions, the central nervous system (CNS) of the three sick pigs had mild lymphoplasmacytic meningoencephalitis (Figure 6.2b) characterized by diffuse gliosis and multifocal discrete perivascular cuffing. Other observed lesions in diseased animals were generalized lymphoplasmacytic myocarditis (4/4), mild lymphoplasmacytic and histiocytic interstitial pneumonia (1/4), mild lymphoplasmacytic nephritis (Figure 6.2c) (3/4) and mild lymphoplasmacytic periportal hepatitis (3/4). Nasal turbinates were examined in only 2 pigs, which displayed mild lymphoplasmacytic rhinitis and mild lymphoplasmacytic periarteritis.

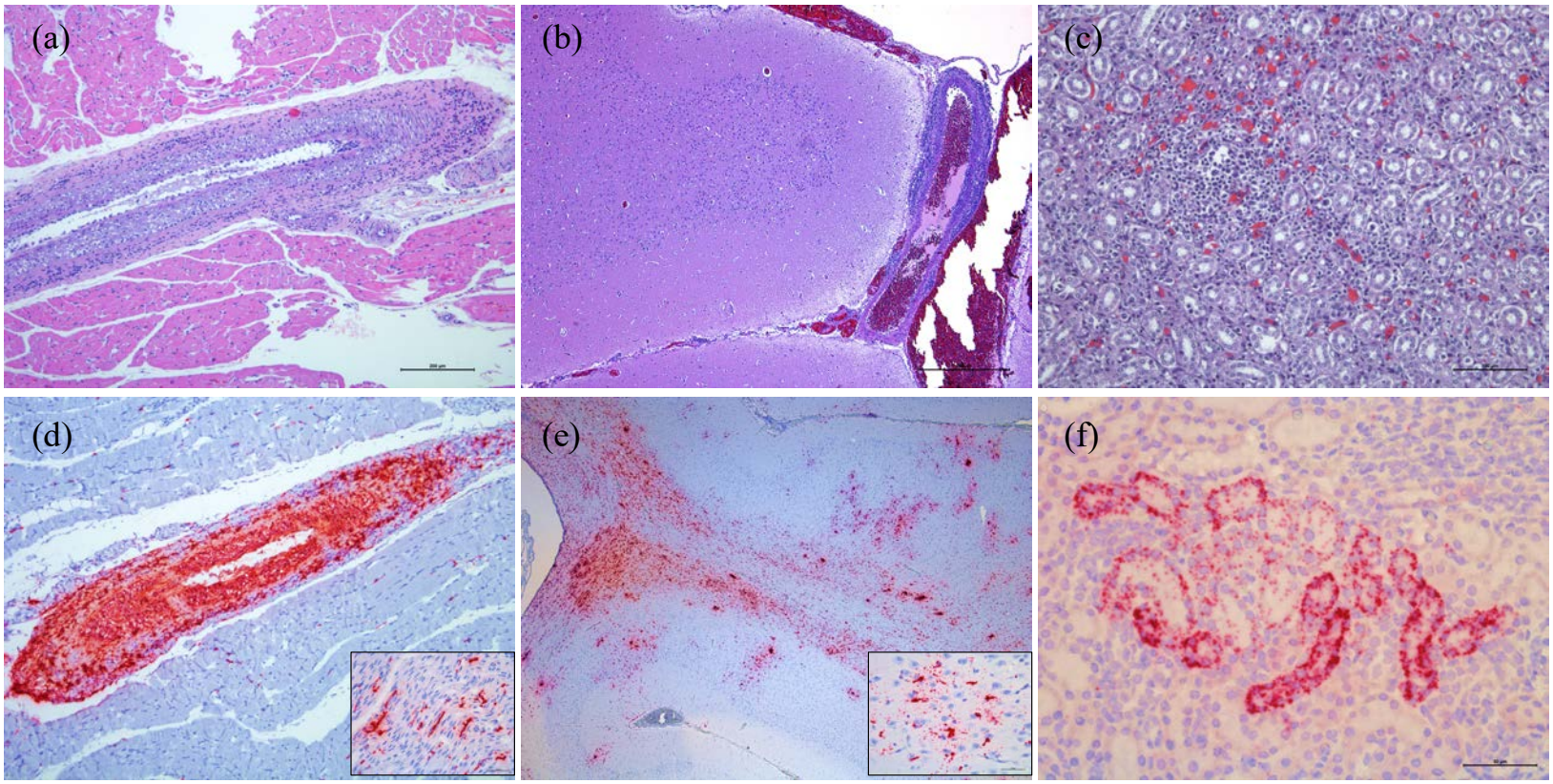

Figure 6.2. Histology (HE stain, a, b, c) and PCV-3 in situ hybridization (d, e, f) results. (a) Heart; intense lymphoplasmacytic periarteritis from case No. 3. (b) Brain; segmental periarteritis in meningeal artery and cortical section without lesions. (c) Kidney; mild interstitial lymphoplasmacytic nephritis from case No. 4. (d) Heart; PCV-3 genome detection in arterial wall and endothelial cells from case No. 3. (Inset) PCV-3 nucleic acid detection in myocardiocyte-like cells of the same animal. (e) High amount of PCV-3 genome in cerebrum white matter and mild-to-moderate in grey matter from case No. 3. (Inset) PCV-3 labelling in neurons of the same case. (f) PCV-3 nucleic acid detection in renal tubular epithelial cells of the same animal.

In the evaluated samples of the four sick animals, all arteries with consistent histologic lesions showed a segmental or circumferential PCV-3 labeling of smooth muscle-like cells, lymphocytes, plasma cells and endothelial cells (Figure 2d). RNA viral detection in lymphoid tissues was observed as stellate-shape cells in germinal centers of lymph nodes (2/3), tonsils (2/3), Peyer's patches (1/3) and white pulp of the spleen (3/4). In the heart, widespread PCV-3 labelling was present in myocardiocyte-like cells (4/4) (Figure 2d-inset). CNS showed extensive labelling in spotted and stellate-like cells, mainly in the white matter but also in multiple foci in the grey matter in neuronal body-like cells (4/4) from cerebellum and cerebrum (Figure 2e and inset). Besides arterial labelling, viral RNA was detected multifocally in the cytoplasm of renal tubular epithelial cells (2/4) (Figure 2f) In the lung, type I pneumocyte-like cells and interstitial macrophage-like cells were labeled (2/4). Scant positivity in the Kupffer's cells (3/4), scattered labelling in intestinal smooth muscle-like cells of the small bowel and spiral colon (1/4), and scant positivity in macrophage-like cells in the nasal turbinate inflammatory infiltrate (2/2) were also observed. Overall, the number of labeled cells were directly proportional to the severity of lesion detected.

Control animals did not show gross or microscopic lesions and no evidence of PCV-3 genome detection was found by ISH.

Furthermore, all animals were evaluated for PCV-2 and PRRSV antigen detection by IHC techniques, yielding negative results by both techniques.

6.4 Discussion

The present report described a wasting disease in post-weaning pigs apparently attributed to PCV-3 infection. Evidence of potential causality was based on presence of multisystemic lesions associated with the presence of viral nucleic acid within them. To date, most of the descriptions of PCV-3 detection in sick animals have been based on real time quantitative PCR (Opriessnig et al., 2020), which makes difficult to establish a sound causality association of the virus with the clinical-pathological condition. Therefore, and considering the widespread nature of PCV-3 over the world, it is urgently needed to establish criteria for a formal case definition of PCV-3 associated disease/s (PCV-3-AD).

A similar situation occurred more than two decades ago with a genetically related circovirus, PCV-2, also ubiquitous. The sole presence of the virus in a pig does not constitute a diagnosis of PCVDs, and the triad 1) clinical signs, 2) moderate to severe histological

lesions and 3) moderate to high amount of PCV-2 in damaged tissues, is required to substantiate a definitive diagnosis (Segalés, 2012). Equivalent criteria have been demonstrated in very few reports for PCV-3, mainly regarding reproductive failure (Palinski et al., 2017; Arruda et al., 2019; Williamson et al., 2019; Study III) and myocarditis and periarteritis in post-weaning pigs (Phan et al., 2016; Arruda et al., 2019). Some descriptions have used ISH to detect PCV-3 genome in different tissues from sick animals, but the amount of viral material detected was low (Phan et al., 2016; Kim et al., 2018a), which poses uncertainty on the real causality of the condition. Arruda et al. (2019) described myocarditis and multi-organic periarteritis associated with a significant amount of PCV-3 genome in 12 cases of post-weaning pigs suffering from different clinical conditions in the USA (Arruda et al., 2019). However, no further cases have been reported around the world to date. Therefore, the cases described here represents the first description of PCV-3-AD in post-weaning pigs in Europe.

Importantly, an abundant amount of PCV-3 genome was found in all 4 diseased pigs described here, including the brain (mainly cerebrum and cerebellum), fact that has not been previously reported in post-weaning pigs (Arruda et al., 2019). Importantly, these animals did not show central nervous system clinical signs despite the mild meningoencephalitis associated with PCV-3. Presence of viral genome in brain, in contrast, has been found in cases of perinatal disease in several reports (Arruda et al., 2019; Williamson et al., 2019; Study III).

Obtained data suggest that PCV-3 may replicate in epithelial cells from the endothelia, as well as renal tubular cells, pneumocytes, smooth muscle cells, myocardial cells, dendritic cells/macrophages, and glial cells. Some of these cell types have already been suggested as supporting viral replication by means of experimental settings (Mora-Díaz et al., 2020). In any case, much more research is needed to establish the pathogenesis of PCV-3 infection in pigs and to elucidate the cells supporting viral replication.

In summary, the present study reports the first documented case of post-weaning disease associated with PCV-3 in wasting pigs in Europe, which was characterized by angiocentric, systemic inflammatory disease.

CHAPTER 7
GENERAL DISCUSSION

General discussion

When the present Thesis was proposed, PCV-3 had been detected worldwide in pigs from different production phases, clinical conditions and at variable frequencies. At that time, most of the published reports where PCV-3 had been detected in diseased animals did not include a control group with healthy age-matched animals to compare with. Such fact complicated the interpretation of the potential role of the virus in disease causality. In such global scenario, the present Thesis was oriented to gain knowledge on the role of PCV-3 on disease occurrence. To reach this goal, the potential association of PCV-3 with different clinical-pathological conditions was analyzed under the scope of four different approaches (Studies I to IV). Overall, studies performed within this Thesis suggest that PCV-3 is a widespread pathogen that can infect pigs at almost all ages, and it can be associated with reproductive/peri-natal disorders and post-natal multisystemic inflammatory disease. However, no evident link with digestive and respiratory conditions was noticed.

In the Study I of this Thesis, the potential association of PCV-3 with respiratory or enteric diseases was assessed. At that time, a few studies reported a putative association between PCV-3 and these pathological conditions. Nevertheless, these works did not investigate the PCV-3 infection in age-matched healthy animals (Zhai et al., 2017; Kedkovid et al., 2018; Shen et al., 2018; Qi et al., 2019). To circumvent such limitation, in Study I, the frequency of PCV-3 in diseased animals was compared to the frequency of viral detection in healthy animals of similar age. Obtained results, however, did not point out an apparent association of the virus, both in frequency of detection or in viral load, with pigs suffering from different types of respiratory or digestive lesions compared to healthy ones. In consequence, taking together all literature available so far regarding this type of disorders, it is considered unlikely that PCV-3 may cause enteric or respiratory disease.

While conducting the Study I of this Thesis, some other publications on potential PCV-3 association with disease were published. Some of them proposed a link of PCV-3 with reproductive disease (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017; Kim et al., 2018a; Tochetto et al., 2018; Zou et al., 2018; Arruda et al., 2019; Deim et al., 2019). Taking into account these data, two more studies were designed. The first one (Study II) was conceived to describe the normal percentage of PCV-3 infection in farms without evident reproductive problems. The second study (Study III) intended to assess the frequency of PCV-3 in cases of overt reproductive disorders in Spain and the possible

association of the viral genome detection within the lesions observed in aborted fetuses or stillborn piglets.

Since some already published studies indicated the presence of the virus in reproductive failure cases (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017; Kim et al., 2018a; Tochetto et al., 2018; Zou et al., 2018; Arruda et al., 2019; Deim et al., 2019), it was very important to establish how often the virus can be found in fetuses and stillborn piglets of farms without such problems, as a potential baseline of the expectable frequency of detection of the virus in such scenario. Results obtained from Study II indicated a fairly high incidence of PCV-3 infection in primiparous sows and their fetuses and stillborn piglets, although with very low viral loads. This was in contrast with those coming from multiparous sows, in which PCV-3 infection was minimal. Based on these results, it is tempting to speculate that the likelihood of occurrence of reproductive problems associated to PCV-3 is higher in primiparous sows rather than in multiparous ones. Such scenario would probably be related with herd immunity, as already discussed in Study II. Regretless, data on the parity number of sows included in Study III were not available.

Since the simple viral detection in animals displaying clinical signs is not sufficient to establish a causality relationship, Study III is especially valuable for the demonstration of PCV-3 genome within the lesions of some of the reproductive failure cases. In fact, high viral load was detected in the tissue pools of fetuses/stillborn piglets in six cases. Among them, the four with highest viral loads (from 106 to 108 copies/mL) showed microscopic lesions and the virus was detected by ISH within such lesions, further supporting the association of disease occurrence with certain viral loads. Similar association of PCV-3 with reproductive losses was also demonstrated by Arruda et al. (2019) and Williamson et al. (2021) in mummified and stillborn piglets, with also high viral loads in both mentioned cases (from 105 to 1012 copies/mL and around 109 copies/mL respectively). Moreover, Williamson et al. (2021) also found the same strong evidence of high amount of virus by ISH within histologic lesions in cases of lactating animals (18-days-old); Arruda et al. (2019) also found viral loads around 108 copies/mL in 1 to 12 day-old piglets.

Indeed, in Study IV, a potential causality association of PCV-3 with a post-weaning condition, based on the consistent detection of viral nucleic acid within systemic inflammatory lesions, was reported. Such evidence was also described by Arruda et al. (2019). In both cases, affected animals showed wasting (poor-doer animals) but, while

Arruda et al. (2019) reported acute deaths and dermatopathy problems, animals included in Study IV displayed wasting and through back ears. Further studies to figure out the frequency and economic impact of this pathologic condition are needed.

The simple detection of a virus in animals or humans displaying clinical signs is not sufficient to establish a causality relationship. According to Koch's postulates, disease causality by a given pathogen would be confirmed if: 1) the pathogen is found in diseased animals and not in healthy ones, 2) the pathogen is isolated from the diseased subjects, 3) the isolated pathogen causes the disease when inoculated in healthy subjects and 4) the pathogen is re-isolated from the inoculated animals and the agent matches with the original pathogen (Koch, 1892; Rivers, 1937). These postulates were very timely and adequate to order the scientific knowledge by late XIX century, but they are very stringent since only unifactorial diseases (those in which the single presence of the pathogen can cause disease in a naïve subject) would be represented under these rules. Nowadays, however, a significant number of pathogens are associated to multifactorial diseases, i.e., when the clinical expression of the disease depends on the coincidence of different risk factors together with the causative agent (Segalés et al., 2013). An example of a virus causing a multifactorial disease from which the causality was under debate for this reason was PCV-2 (Segalés et al., 2013). However, other pathogens such as Mycoplasma hyopneumoniae (Mhyo) which together with other agents is the main etiological agent of enzootic pneumonia (EP) (Sibila et al., 2012; Maes et al., 2018), colibacillosis where the severity of the disease depends on the strain virulence but also the environmental conditions and host factors (Fairbrother and Nadeau, 2019), and even PRRSV, which may fail to induce overt respiratory disease under experimental circumstances (Van Reeth et al., 1996), are considered as multifactorial diseases (Van Gucht et al., 2004). Similar to the previously mentioned pathogens, PCV-3 infection is not able to fulfill Koch's postulates, mainly because it can be detected in diseased as well as in healthy pigs (Stadejek et al., 2017; Zhai et al., 2017; Klaumann et al., 2018a; Klaumann et al., 2018b; Klaumann et al., 2019; Franzo et al., 2018; Ye et al., 2018; Saporiti et al., 2020), among other reasons. Moreover, when the present Thesis started, all the published attempts to isolate the virus from damaged PCV-3 PCR positive tissues were unsuccessful (Faccini et al., 2017; Palinski et al., 2017). Very recently, two research groups have been able to isolate PCV-3 (Oh and Chae, 2020; Mora-Díaz et al., 2020), but so far, no more research groups have achieved it, indicating the difficulty of this procedure. On one hand, a South Korean research group isolated the virus from a lymph node of a clinically healthy pig in

primary porcine kidney cells with no cytopathic effect, but the presence of PCV-3 was confirmed by ISH (Oh and Chae, 2020). On the other hand, Mora-Díaz et al. (2020) could isolate PCV-3 in PK-15 cells from fetal tissues of farms experiencing reproductive losses. Importantly, when this latter virus isolate was used to inoculate nursery piglets, they developed mild histologic lesions (multisystemic inflammation, such as myocarditis, interstitial nephritis, and arteritis and periarteritis in heart, kidney and intestinal serosa) without showing overt clinical signs (Mora-Díaz et al., 2020). Up to now, two other PCV-3 experimental infections, using tissue homogenate or an infectious clone as inoculum have been published (Jiang et al., 2019; Temeeyasen et al., 2021). Temeeyasen et al. (2021) challenged animals with a tissue homogenate and found similar results as Mora-Díaz et al (2020); the piglets showed histologic lesions, but no clinical disease was observed. On the other hand, Jiang et al. (2019), using the infectious clone, indicated that the inoculated pigs showed a PDNS-like condition, fever, anorexia, coughing, sneezing and diarrhea. However, the histopathological description of the kidney lesions did not fit with those of classical PDNS lesions (systemic necrotizing vasculitis and fibrino-necrotizing glomerulonephritis) (Segalés et al., 2012) and the PCV-3 IHC images published of these tissues are difficult to be interpretated (Jiang et al., 2019). In none of these experimental inoculations, re-isolation of PCV-3 from affected tissues has been attempted or reported. Therefore, taking all available knowledge into account, PCV-3 infection is not apparently able to fulfil Koch's postulates.

Based on the widespread nature of PCV-3 in healthy animals, it is very likely that its potential pathogenicity might be triggered by other infectious and/or non-infectious factors. This would be the scenario of a multifactorial disease in which PCV-3 should be a necessary factor, but not sufficient to cause overt disease. Therefore, evidence so far suggests that PCV-3 is associated with disease causality under certain, not yet determined conditions. Further research on these conditioning factors might be relevant in the future to assess the relative importance of this novel virus among existing swine pathogens. However, considering the current knowledge on PCV-3, its association with disease might be supported by the fulfilment of the Evans' postulates (Evans, 1976). These postulates take into consideration both experimental and epidemiological factors to explain causality by an infectious or not infectious element. Table 7.1 summarizes Evans' postulates and their potential fulfilment by PCV-3.

Table 7.1. The fulfillment of Evans' postulate (Evans, 1976) with PCV-3.

Evan's postulates	**Fulfilment Yes / No**	**Evidence**	**References**
1 - Prevalence of the disease should be significantly higher in those exposed to the putative cause than in cases controls not so exposed	Yes	Although the prevalence of PCV-3 in diseased and healthy animals are variable among published studies, pigs with higher viral loads are those showing clinical disease or lesions linked to PCV-3	Study III Mora-Díaz et al., 2020 Temeeyasen et al., 2021 Jiang et al., 2019
2 - Exposure to the putative cause should be present more commonly in those with the disease than in controls without the disease when all risk factors are held constant	Yes	Exposure has only been tested under experimental conditions, and inoculated animals develop microscopic lesions not being developed by non-inoculated animals	Mora-Díaz et al., 2020; Temeeyasen et al., 2021 Jiang et al., 2019
3 - Incidence of the disease should be significantly higher in those exposed to the putative cause than in those not so exposed as shown in prospective studies	Yes	Microscopic lesions were only developed by those animals exposed to the virus in experimental infections; not possible to assess based on natural occurring infections	Mora-Díaz et al., 2020; Temeeyasen et al., 2021 Jiang et al., 2019
4 - Temporally, the disease should follow exposure to the putative agent with a distribution of incubation periods on a bell shaped curve	Unknown	No sufficient data do exist to date to assess this postulate	None
5 - A spectrum of host responses should follow exposure to the putative agent along a logical biologic gradient from mild to severe	Yes	Under natural conditions, PCV-3 can be associated with clinical conditions with moderate to high amount of viral genome, while animals with low amount of viral nucleic acid do not display lesions or clinical signs	Studies III and IV Phan et al., 2016 Arruda et al., 2019

Table 7.1. (Continuation) - The fulfillment of Evans' postulate (Evans, 1976) with PCV-3.

6 - A measurable host response following exposure to the putative cause should regularly appear in those lacking this before exposure (i.e., antibody, cancer cells) or should increase in magnitude if present before exposure; this pattern should not occur in persons so exposed	Yes	Evidence of humoral immune response (IgM and/or IgG) in experimentally inoculated animals as well as in naturally infected animals	Palinski et al., 2017 Mora-Díaz et a., 2020 Temeeyasen et al., 2021
7 - Experimental reproduction of the disease should occur in higher incidence in animals or man appropriately exposed to the putative cause than in those not so exposed; this exposure may be deliberate in volunteers, experimentally induced in the laboratory, or demonstrated in a controlled regulation of natural exposure	Yes	Correspondence with postulate No. 2, in terms of microscopic lesion reproduction	Mora-Díaz et al., 2020; Temeeyasen et al., 2021 Jiang et al., 2019
8 - Elimination or modification of the putative cause or of the vector carrying it should decrease the incidence of the disease (control of polluted water or smoke or removal of the specific agent)	Yes	Correspondence with postulates No. 2 and 7, since negative control animals did not develop microscopic lesions linked to PCV-3; expected under field conditions, but not demonstrated.	Mora-Díaz et al., 2020; Temeeyasen et al., 2021 Jiang et al., 2019
9 - Prevention or modification of the host's response on exposure to the putative cause should decrease or eliminate the disease (immunization, drug to lower cholesterol, specific lymphocyte transfer factor in cancer)	Unknown	No particular actions have been yet designed to counteract the effect of PCV-3	None
10 - The whole thing should make biologic and epidemiologic sense	Yes	Both experimental and natural infections showing the presence of PCV-3 genome in lesions support potential causality; low viral loads are related with no lesions and no clinical signs	Studies I, II, III and IV Arruda et al., 2019 Mora-Díaz et a., 2020 Temeeyasen et al., 2021

All in all, today's evidence suggests PCV-3 as a potential pathogen causing reproductive problems in sows (abortion, mummified, stillborn and weak-born piglets) and systemic inflammatory disease in pre- and post-weaning pigs. However, the frequency of these clinical problems under field conditions is unknown and, therefore, no data on their economic impact in the swine industry do exist. It may happen as well that the putative disease conditions linked to PCV-3 may occur in the framework of non-etiologically diagnosed problems or diagnosed as other well-established diseases. This latter possibility would open the avenue for studying the importance of PCV-3 in co-infection with other pathogens.

Although the abovementioned strong evidence of PCV-3 association with reproductive and systemic disorders, there is not yet a proposal on diagnostic criteria for these conditions. At this point, it would be important to provide an ordered, concise, and systematic approach for diagnosing disorders apparently caused by PCV-3. This way, it will be easier to identify the cases and, thus, know how spread it is and, in a long term scale, measure the impact of it in the swine production. Considering the epidemiologic similarities between PCV-2 and PCV-3 (multifactorial nature, ubiquitous distribution of the virus and detection in healthy and diseased animals), the existing PCV-2 diseases (PCVD) case definition could be of a great help to propose formal criteria for PCV-3 associated diseases (PCV-3-AD). In the case of PCV-2-SD, the three accepted criteria are (Segalés and Domingo, 1999; Sorden et al., 2000): 1) presence of compatible clinical signs, mainly wasting, 2) presence of moderate to severe histological lesions in lymphoid tissues (lymphocytic infiltration and histiocytic infiltration), and 3) detection of moderate to high amount of PCV-2 within such lesions. Considering the existing clinical, pathological, and virological information derived from this thesis (especially in Studies III and IV) and other studies (Arruda et al., 2019, Kim et al., 2018a, Phan et al., 2016), it is proposed that PCV-3 can be associated to two major disease outcomes: PCV-3-reproductive disease (PCV-3-RD) in sows and their offspring, and PCV-3-systemic disease (PCV-3-SD) in pre- and post-weaning pigs. Following the known PCV-2 disease case definition, the diagnostic criteria proposed for PCV-3-AD are displayed in Table 7.2.

Table 7.2. Proposed diagnostic criteria for the individual case definition of PCV-3 associated diseases (PCV-3-AD).

PCV-3- AD	Main clinical sign	Individual diagnostic criteria
PCV-3-reproductive disease (PCV-3-RD)	Late abortion, malformations, mummified fetuses, stillborn fetuses, weak-born piglets	1) Late reproductive problems and higher perinatal mortality 2) Systemic lymphoplasmacytic to lymphohistiocytic perivascular inflammation in fetal tissues 3) Moderate to high amount of PCV-3 genome in damaged tissues
PCV-3-systemic disease (PCV-3-SD)	Wasting, weight loss, ill thrift and/or poor-doer	1) Weight loss, rough hair 2) Systemic lymphoplasmacytic to lymphohistiocytic perivascular inflammation 3) Moderate to high amount of PCV-3 genome in damaged tissues

In PCV-2 infection, viral loads of PCV-2-SD affected pigs are particularly high when compared to subclinically infected ones (Olvera et al., 2004; Brunborg et al., 2007), and a threshold of 10^7 copies of PCV-2 DNA copies per mL of serum was proposed to differentiate diseased versus non-clinically diseased animals. The same situation seems to apply in those cases in which PCV-3 might be the potential cause of disorders, since tissues from sick animals showed high viral loads, ranging from 10^6 to 10^{12} copies/mL of macerated or pool of tissue (Study III, Faccini et al., 2017; Palinski et al., 2017; Arruda et al et al., 2019). A fact that may support this hypothesis is the low viral loads usually found in healthy pigs infected with PCV-3 (Kedkovid et al., 2018a; Klaumann et al., 2019; Saporiti et al., 2020). However, studies published so far detected PCV-3 in many different matrices (serum as well as different tissues), which makes difficult to establish an equivalence of viral loads among them (Wozniak et al. 2020).

In the first study of this Thesis, the PCV-3 load detected in sera from healthy animals and in animals with respiratory and digestive clinical signs was low (from 10^3 to 10^5 copies /mL). Unfortunately, fresh tissues were not available to be evaluated. Nevertheless, according to what has been described (Wozniak et al., 2020), the viral load in tissues from affected pigs would probably be higher than in sera. This idea would be in line with results from Study II, where primiparous sows showed low amount of virus in sera (range from 10^3 to 10^5 copies/mL) while some of their respective stillborn piglets and mummifies fetuses harbored a higher amount of virus in lung and brain (range from 10^3 to 10^{10} copies/mL). This high viral load in some particular fetuses from primiparous sows would suggest that PCV-3 was the causal agent of fetal death; however, since no formalin-fixed, paraffin-embedded tissues were available for histopathology or ISH it cannot be ascertained. Anyway, it seems that the percentage of losses due to this fact was not enough to impact the overall reproductive parameters of the farm. On the contrary, in Study III, a putative association between high viral load and presence of histologic lesions was observed. Indeed, the fetuses with the highest amount of virus (from 10^6 to 10^8 copies/mL) showed the PCV-3-RD associated histologic lesions. In Study IV, whereas animals suffering from PCV-3-SD showed a huge amount of virus observed by ISH in tissues, the healthy ones did not show any trace of viral genome in any organ. Presumably, the PCV-3 load in serum and tissue samples of these affected animals should also be very high.

To detect the presence of PCV-3 genome in histologic lesions, the ISH has been the most used technique so far (Phan et al., 2016, Kim et al., 2018a, Arruda et al., 2019, Studies III and IV). However, the ISH is an expensive and time demanding technique that prevents to be stablished as a routine diagnostic. To solve the barrier for a proper PCV-3 diagnostic approach, the development of a cheaper technique to detect the virus within the damaged tissues is essential. An alternative, as for PCV-2, could be IHC. However, the lack of commercial PCV-3 specific antibodies makes it, for the moment, difficult. The intrinsic limitations of generating specific diagnostic reagents and tests are also on the side of immunomonitoring, fact that implies the current paucity of knowledge regarding the antibody response of pigs upon infection with PCV-3, developing or not clinical disease. Although some laboratories have developed reagents and techniques (mainly ELISA), its usage is limited to their research only (Li et al., 2018; Zhan et al., 2019). In consequence, the knowledge of PCV-3 in terms of immunity and pathogenesis is still very scarce and

depends on the development of good diagnostic techniques to cover these points in future studies.

Another issue to be elucidated is the impact of PCV-3-AD in the context of the swine production. Most of the studies published to date were focused on the description of PCV-3 infection under different scenarios and pathological conditions. However, once an association has been stablished and diagnostic criteria have been proposed, the interest of swine producers, clinicians and scientists would be to assess the economic impact of these diseases. Interestingly, in Study III, the prevalence of PCV-3 PCR positivity in the reproductive cases tested was around 34%, being much higher than those for PCV-2, PRRSV and PPV, very well-established reproductive pathogens, with 10%, 7.5% and 0%, respectively. This result does not allow establishing the full causality of the problems but supports the inclusion of PCV-3 as a potential cause of reproductive losses.

Another aspect that deserves further investigations, is the relevance of PCV-3 under subclinical infection scenarios. In the present Thesis, such scenario has been explored in Studies I and II. At least for another PCV, PCV-2, the subclinical infection is the most common PCVD and its impact was properly measured only after the spread usage of vaccination; PCV-2 immunization demonstrated an improvement of the productive parameters, especially in average daily weight gain, even when no clinical signs where observed (Kurmann et al., 2011; Young et al., 2011). Taking in account that PCV-3-AD comprise apparently new diseases with unknown economic impact, the development of a vaccine it not yet justified.

PCV-3 has been found in many studies in co-infection with other pathogens such as PCV-2, PRRSV, APPV, PPV, CSFV, PEDV, *Mycoplasma hyorhinis* and *Mycoplasma hyopneumoniae,* among others (Chen et al., 2017; Phan et al., 2017; Kedkovid et al., 2018b; Kim et al., 2018a; Zhao et al., 2018; Wozniak et al., 2019; Williamson et al., 2021). The existence of such mixed infections in diseased animals emphasizes the need to study the pathogenesis of PCV-3 infection in presence and absence of other pathogens. Mirroring again at PCV-2, co-infection of this virus with other pathogens, such as PRRSV, PPV and *M. hyopneumoniae* can lead to more severe disease presentation under field (Opriessnig & Halbur, 2012) and experimental (Tomás et al., 2008) conditions. The impact of PCV-3 co-infection with other agents has not been explored yet.

PCV-3 genetic characterization was performed in most of the studies of the present thesis (Studies I, II and III). In Study I, samples were classified following the newest

proposal by that time (Fux et a., 2019) in subtypes (PCV-3a1, PCV-3a2, PCV-3b1 and PCV-3b2). However, the increase of sequences available in GenBank and their phylogenetic analyses allowed the description of a PCV-3 mutation rate (10^{-5} to 10^{-6} substitutions/site/year) (Franzo et al., 2019). This rate would be considered low when compared to PCV-2 (10^{-3} substitutions/site/year) (Franzo et al., 2016) or other ssDNA virus such as TTSuV or PPV1 (both with 10^{-4} substitutions/site/year) (Karuppannan and Opriessnig, 2018). Thus, with such low mutation rate, it was expectable to find low genomic variability across the performed studies. In fact, nowadays, the most novel proposal on PCV-3 genotyping includes one single genotype, the PCV-3a (Franzo et al., 2020). Hence, this genotype proposal was used in Studies II and III of this Thesis. Samples from Study I could have not been analyzed following Franzo et al. (2020) proposal as the maximum length retrieved in this study was 1,237 nt of the complete genome and 344 nt of ORF2 gene. To ascertain to which genotype would belong those samples from Study I, two further phylogenetic trees including the samples from all the studies of the Thesis were constructed (Figure 7.1, using the maximum length of the whole genome, and Figure 7.2, using the maximum length of the ORF2 gene retrieved in all studies). As expected, sequences from Study I clustered among the sequences from Studies II and III, being all of them PCV-3a (Franzo et al., 2020). No sequencing analyses were done in Study IV.

Despite the low mutation rate of PCV-3, it is important to keep analyzing phylogenetically the samples found in the field. As for example, in Study III, one of the analyzed sequences was in the limit of the proposed classification of PCV-3a genotype (Franzo et al., 2020) and, therefore, it is worthy to keep an eye on possible changes. Noteworthy, so far there is no suggestion of a potential relation of the genotype with any differential clinical outcome or virulence.

In conclusion, this Thesis provided relevant knowledge on clinical, pathological and virological data of PCV-3 infection in swine, thus further suggesting PCV-3 as a virus with pathogenic potential, implying the need to standardize diagnostic criteria for at least reproductive and pre-/post-weaning disorders.


Tree scale: 0.01
MW167066.1 Study III
MW167063.1 Study III
MW167064.1 Study III
MK904831.1 Study I
MK904829.1 Study I
MK904830.1 Study I
MK904828.1 Study I
MW167067.1 Study III
MW167065.1 Study III
MW167068.1 Study III
MT350520.1 Study II
MT350518.1 Study II
MT350523.1 Study II
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Figure 7.1. Phylogenetic tree of PCV-3 sequences from Studies I, II and III of this Thesis. The tree was constructed based the 1,237nt (maximum length retrieved in Study I) of samples from this thesis using Hasegawa-Kishino-Yano (HKY) model with 1,000 bootstraps replicates. Samples were colored by Study (Study I in green, Study II in blue and Study III in red).

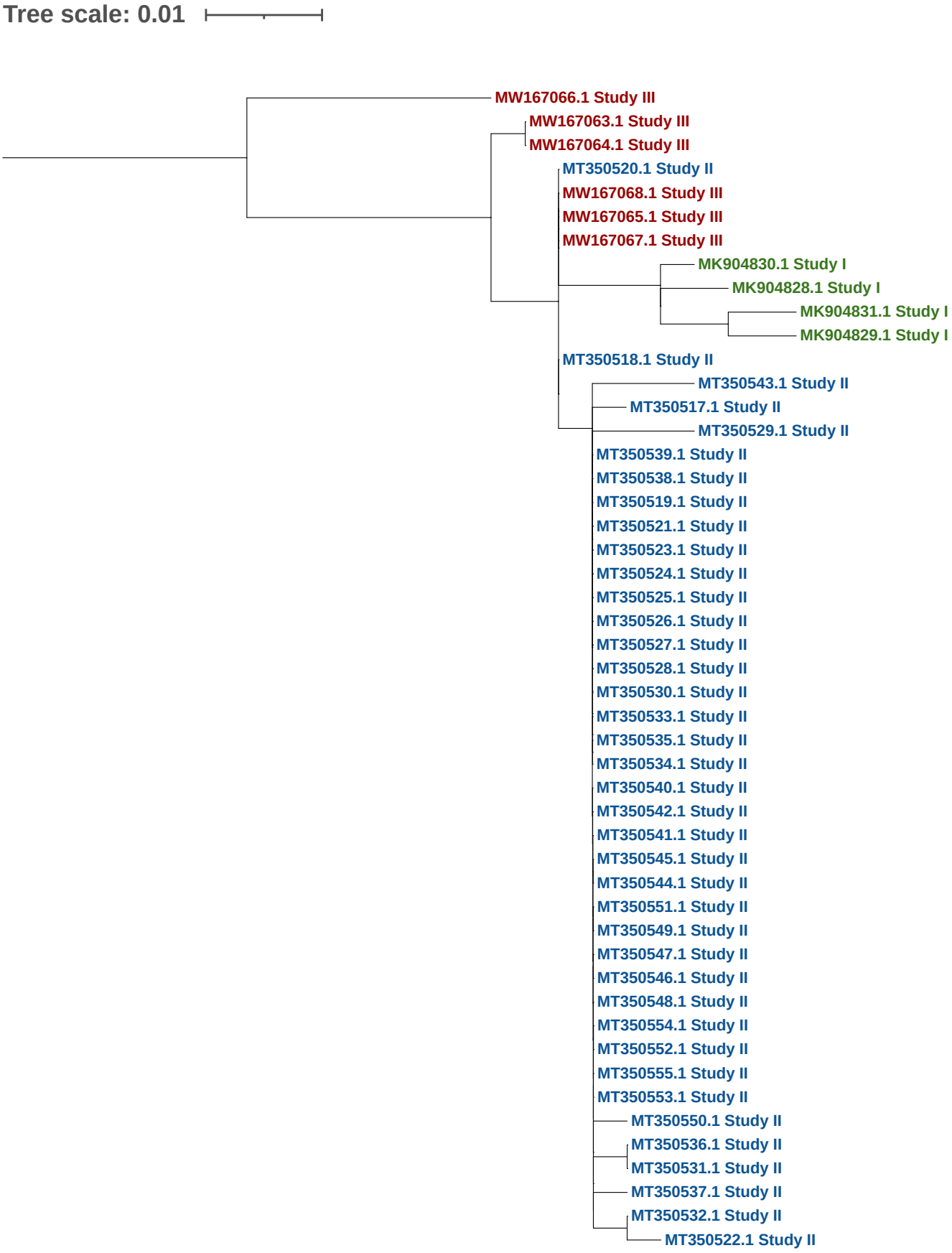
Tree scale: 0.01
MW167066.1 Study III
MW167063.1 Study III
MW167064.1 Study III
MT350520.1 Study II
MW167068.1 Study III
MW167065.1 Study III
MW167067.1 Study III
MK904830.1 Study I
MK904828.1 Study I
MK904831.1 Study I
MK904829.1 Study I
MT350518.1 Study II
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Figure 7.2. Phylogenetic tree of PCV-3 ORF2 sequences from Studies I, II and III of this Thesis. The tree was constructed based the 344 nt (maximum length retrieved of ORF2 in Study I) of ORF2 from samples retrieved in this thesis using Kimura 2-parameter (K2) model with 1,000 bootstraps replicates. Samples were colored by Study (Study I in green, Study II in blue and Study III in red).

CHAPTER 8
CONCLUSIONS

Conclusions

1. PCV-3 frequency of detection in serum was similar in diseased animals displaying well characterized histologic lesions of respiratory and digestive disorders and in the healthy analyzed animals, thus, not pointing out a causal association of PCV-3 with these conditions.

2. PCV-3 can cause intrauterine infection in absence of overt reproductive problems. A higher frequency of viral infection was found in primiparous sows compared to multiparous ones, as well as in mummified fetuses when compared to stillborn piglets.

3. PCV-3 loads in tissues of fetuses from multiparous sows were lower than those in fetal tissues from primiparous ones.

4. PCV-3 genome can be detected in association to cases of reproductive failure; viral nucleic acid detected by *in situ* hybridization was located within histologic lesions observed in aborted fetuses with high viral loads detected by real time quantitative PCR.

5. PCV-3 nucleic acid can be detected in association to cases of wasting disease in post-weaning pigs; high amount of viral nucleic acid detected by *in situ* hybridization was found within histologic lesions.

6. PCV-3 infection linked to histological lesions of systemic lymphoplasmacytic and/or lymphohistiocytic perivascular multisystemic inflammation in reproductive and post-weaning wasting disorders suggest a potential disease causality, thus, implying the need to standardize diagnostic criteria.

7. PCV-3 genome sequences analyzed in the studies of this Thesis showed low genomic variability and were all classified as PCV-3a.

CHAPTER 9
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