Universitat Autònoma de Barcelona

Molecular epidemiological studies of *Porcine circovirus 3*, a novel virus identified in domestic pig and wild boar

Francini Klaumann

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PhD Thesis

Bellaterra, 2018

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Tesis doctoral presentada por **Francini Klaumann** para acceder al grado de Doctor en el marco del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, bajo la dirección del Dr. **Joaquim Segalés Coma**, del Dr. **José Ignacio Núñez Garrote** y de la Dra. **Florencia Correa Fiz**.

Bellaterra, 2018

El Dr. JOAQUIM SEGALÉS COMA, profesor titular del Departament de Sanitat i d' Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona (UAB) e investigador del Centre de Recerca en Sanitat Animal (CReSA-IRTA); el Dr. JOSÉ IGNACIO NÚÑEZ GARROTE, investigador del CReSA-IRTA; y la Dra. FLORENCIA CORREA-FIZ, investigadora del CReSA-IRTA.

Certifican:

Que la memoria titulada **"Molecular epidemiological studies of *Porcine circovirus 3*, a novel virus identified in domestic pig and wild boar"** presentada por Francini Klaumann para la obtención del grado de Doctor en Medicina y Sanidad Animal, se ha realizado en dichos centros y bajo nuestra supervisión.

Para que conste los efectos oportunos, firman la presente en Bellaterra (Barcelona), en 19 de septiembre de 2018.

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Memoria presentada por **Francini Klaumann**
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The work performed in this PhD Thesis has been funded by:

-CAPES Foundation, Ministry of Education of Brazil.

Ao meu herói Rogério e ao meu anjo Nelita
Pelas asas que me permitiram voar

AGRADECIMIENTOS

"We are architect of our destiny" (Albert Einstein).

Falando como a pessoa cética que eu sou, diria que em minha vida nunca existiram milagres ou qualquer força divina que fizesse eu ter chego tão longe. Existiram sim sonhos, existiram pessoas que sonharam comigo e que acreditaram que toda a loucura sonhada pudesse ser realizada. Minha base regida por três pilares foi essencial para concluir essa etapa na minha vida. Mãe, obrigada por ser essa mulher tao inspiradora e por ter abdicado de tanto durante todos esses anos, muitas vezes dos teus próprios sonhos para que eu pudesse viver os meus. Meu pai, o homem mais bondoso que eu já conheci, obrigada por dar-me a oportunidade de sonhar e por toda essa sabedoria que compartilhas comigo, és uma inspiração. Ana, luz da minha vida, eu te agradeço por escutar-me, por ter sido essa grande amiga e ter melhorado a pessoa que eu sou. Amo cada um de vocês com todo meu coração.

Agradeço ao governo brasileiro democraticamente eleito, que em 2015 concedeu-me a bolsa de estudos. Obrigada à equipe que idealizou o Programa Ciência sem Fronteiras e aos avaliadores do projeto por terem dado a oportunidade de contribuir com essa Pátria Amada, idolatrada, Brasil.

De manera especial, agradezco a mis directores de tesis. Quim, gràcies per aquesta font diària de positivisme i entusiasme, per la teva capacitat de treball i resolució, per més d'una centena d'idees compartides, per dirigir aquesta tesi i per tot l'aprenentatge què m'has proporcionat. Gràcies per haver sigut aquella persona amable. Al meu jefe estimat, has canviat la meva vida per complert, no hi ha suficients paraules per agrair-t'ho. I, per últim: quina pressió, jefe! Flor, gracias por haber formado parte de este período. Gracias por ayudarme a "plantar" mis primeros árboles, por todas las correcciones en

ese trabajo, consejos, gentileza y sobre todo por creer que esto sería posible. Ignacio, gracias por aceptar el desafío de dirigir la tesis y gracias por todo el aprendizaje que he obtenido durante todo este periodo de esfuerzo.

A familia Cresa, en especial a los miembros del PCV-3 team. Anna Llorens, la base de todo mi conocimiento laboral, gracias por prestarme tus manos mientras las mías aún no eran independientes; a Eva por los pequeños grandes consejos que agilizaron muchas etapas de mi tesis.

A nuestro querido coautor Óscar Cabezón y el equipo de fauna salvaje por todo el trabajo que vosotros habéis hecho para la obtención de las muestras.

A Giovanni per tutto l'aiuto nell'interpretazione dei risultati, dei protocolli e per la pazienza nell'insegnarmi. Grazie per essere parte di questa tesi, per l'amicizia costruita e per essere una persona così gentile, sicuramente la più gentile che ho incontrato in tutta la mia vita.

¡A todos los colaboradores de nuestros estudios, Muchas gracias!

My family called ‘Becarios’. I had such a great time with you sharing laughter, problems and confidences. You cannot imagine the importance of each one of you has in my life. I remember that at the very beginning I believed that it would be possible not to create strong friendship bonds; it would make easier the moment to leave. How can I do now? I will keep you in my heart and I hope that one day our paths cross again.

To all of you, my eternal gratitude!

LIST OF ABBREVIATIONS

ADV	Aujeszky's disease virus
AIDS	Acquire immune deficiency síndrome
AI-AO	All in-all out management
Bp	Base pairs
BHQ1	Black Hole Quencher-1 deoxythymidine
Cap	capsid associated protein
CP	*Clostridium perfringens*
CSFV	*Classical swine fever virus*
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTP	Deoxinucleósido trifosfato
Dpi	days post-infection
dsDNA	Double stranded DNA
EC	*Escherichia coli*
ELISA	Enzyme- Linked ImmunoSorbent Assay
Ery	*Erysipelothrix rhusiopathiae*
EU	Enzymatic unit
EVD	Ebola virus disease
FAM	6- carboxy-fluorescein
HEV	*Hepatitis* E virus
HIV	Human immunodeficiency virus
IAV	*Influenza A virus*
ICTV	International Commitee on the Taxonomy of Viruses
IHC	Immunohistochemistry
IPC	Internal positive control
IPMA	Immunoperoxidase monolayer assay
ISH	*in situ* hybridization
MERS-CoV	Middle East Respiratory Syndrome- Coronavirus
Mhyo	*Mycoplasma hyopneumoniae*
NGS	Next generation sequencing
NS	Non-structural protein
nt	Nucleotide
ORF	Open reading frame
Ori	Origin of replication
PBS	Phosphate-buffered saline

PBoV	*Porcine bocavirus*
PCR	Polymerase chain reaction
PCV	*Porcine circovirus*
PCVD	Porcine circovirus disease
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 disease
PCV-3	*Porcine circovirus 3*
PCVD	Porcine circovirus disease
PDNS	Porcine dermatitis and nephrophathy syndrome
PDCoV	Porcine delatacoronavirus
PEDV	*Porcine epidemic diarrhea virus*
PK-15	Porcine kidney cells
PKB	*Porcine kobovirus*
PMWS	Post-weaning multisystemic wasting syndrome
PPV	*Porcine parvovirus*
PRRSV	*Porcine respiratory and reproductive syndrome virus*
PRV	*Porcine pseudorabies virus*
PSV	*Porcine sapelovirus*
PToV	*Porcine torovirus*
qPCR	Real-time quantitative PCR
RCA	Rolling circle amplification
RCR	Rolling circle replication
RDI	Relative density index
Rep	Replication associated protein
RF	Replicative form
RNA	Ribonucleic acid
SIV	*Swine influenza virus*
ssDNA	Single stranded DNA
ST	Swine testicles cells
TAE	Tris-acetate-EDTA
TTSuV	*Torque teno sus virus*
ZIKV	*Zika virus*
USA	United States of America

ABSTRACT

Porcine circovirus 3 (PCV-3) is a recently discovered circovirus species found in domestic pigs and wild boar. The virus was found in 2016, through metagenomic sequencing approach, in animals affected by reproductive failure, cardiac and multisystemic inflammation. Since then, the virus has been described in pigs with different clinical/pathological presentations as well as in healthy ones, with a widespread circulation. Therefore, the main objective of this Thesis was to gain insights into the molecular epidemiology of PCV-3 in samples from domestic pigs and wild boar from Spain.

In the first study, the presence of PCV-3 in the Spanish pig population was retrospectively evaluated from 1996 to 2017 in sera from animals of different production phases and clinical/pathological conditions. The detection of PCV-3 genome in such samples was attempted by PCR and partial genome sequences were obtained from selected PCV-3 positive samples from different years. Compiled data confirmed that PCV-3 has been circulating in the Spanish pig population since 1996. The overall frequency of PCV-3 PCR positive samples in the study period was 11.47% (75 out of 654). Phylogenetic analysis of the PCV-3 obtained sequences showed high identity with the already known PCV-3 sequences, with low variations among years. Although the available information was limited, PCV-3 did not appear to be linked to any specific pathological condition or pig age-group.

The second study of this Thesis aimed to assess the dynamics of PCV-3 infection by means of PCR in serum. A total of 152 pigs from 4 different healthy farms, which were sampled longitudinally five or six times from 2-4 weeks of age until the end of the fattening period, were analyzed. PCV-3 genome was found in pigs from all tested ages and farms; few animals had an apparent long-term infection during a period ranging from 4 to 23 weeks.

Phylogenetic analysis showed high similarity among the obtained sequences and with available PCV-3 genomes from different countries. Results confirmed that PCV-3 circulated in all studied farms from Spain, suggesting that infection is probably widespread in the country. Most pigs got infection during their life, although PCV-3 did not appear to circulate mostly at any specific age.

In the third study, the frequency of PCV-3 infection was retrospectively assessed in Spanish wild boar from 2004 to 2018, as well as in captured and re-captured animals (at least two times in a time period of 1 month to 1 year). Obtained results confirmed the susceptibility of wild boar to the virus, showing high frequency of PCV-3 detection (221 out of 518, 42.66%) and demonstrating circulation at least since 2004. Compiled data suggests that PCV-3 is apparently able to cause persistent infection, since 5 out of 10 PCV-3 PCR positive captured/re-captured boars showed positivity in samplings separated for more than 5 months. The frequency of PCV-3 genome was also investigated for the first time in different tissue samples and feces, where all tested tissue types' harbored PCV-3 genome. The highest percentage of PCR positivity was found in submandibular lymph node, tonsil, lung, liver, spleen and kidney. The amount of DNA in all tested PCV-3 PCR positive samples was moderate to low. All partial and complete PCV-3 sequences obtained from wild boar displayed high nucleotide similarity (>98%).

In conclusion, the obtained results of this Thesis provide relevant data on the epidemiology of this novel virus, PCV-3, in both domestic pig and wild boar, which appear to be widespread. Moreover, the phylogenetic information suggests low genetic variability of PCV-3, in contrast with other single stranded-DNA viruses.

RESUMEN

El *Circovirus porcino 3* es un virus descubierto recientemente en cerdos domésticos y jabalíes. El virus fue hallado por primera vez en 2016 mediante estudios metagenómicos, concretamente en animales afectados por fallo reproductivo, e inflamación cardíaca y multisistémica. Desde entonces, el virus se ha descrito circulando de forma generalizada tanto en animales con diferentes presentaciones clínico/patológicas como en cerdos sanos. Por lo tanto, el objetivo principal de esta Tesis fue generar nueva información sobre la epidemiología molecular del PCV-3 en muestras de cerdos domésticos y jabalíes en España.

En el primer estudio, la presencia de PCV-3 en la población porcina española se evaluó retrospectivamente de 1996 a 2017 en sueros de animales de diferentes fases de producción y condiciones clínico/patológicas. La detección del genoma de PCV-3 en estas muestras se realizó mediante PCR y secuenciación parcial del genoma. Los datos obtenidos confirmaron que PCV-3 ha estado circulando en la población porcina española desde el año 1996. La frecuencia global de muestras PCR positivas para PCV-3 en el período de estudio fue 11.47% (75 de 654). El análisis filogenético de las secuencias obtenidas de PCV-3 mostró una alta identidad con las secuencias de PCV-3 ya conocidas, con mínimas variaciones entre años. Aunque la información obtenida fue limitada, la presencia de PCV-3 no pareció estar relacionada con ninguna condición patológica específica ni asociada a ninguna fase de producción del cerdo.

En el segundo estudio de esta Tesis se evaluó la dinámica de la infección por PCV-3. Para ello se analizaron mediante PCR los sueros de 152 cerdos de 4 granjas de alto estatus sanitario y sin problemas clínicos. Los animales fueron monitorizados longitudinalmente cinco o seis veces desde las 2 a 4 semanas de edad hasta el final de la fase de engorde. El genoma del PCV-

3 se detectó en cerdos de todas las edades y granjas evaluadas; algunos animales presentaron una aparente infección a largo plazo durante un período que varió de 4 a 23 semanas. El análisis filogenético mostró una gran similitud entre las secuencias obtenidas y los genomas de PCV-3 de diferentes países disponibles en las bases de datos. Los resultados confirman que PCV-3 circuló en las granjas estudiadas en España, lo que sugiere que la infección probablemente sea generalizada en el país. La mayoría de los cerdos se infectaron durante su vida productiva, aunque no se encontró asociación con una edad específica.

En el tercer estudio, se verificó la frecuencia retrospectiva de la infección por PCV-3 entre 2004 y 2018, así como en una población española de jabalíes capturados y recapturados (al menos dos veces en un período de un mes a un año). Los resultados obtenidos confirmaron la susceptibilidad del jabalí a la infección por el virus, mostrando alta frecuencia de detección de PCV-3 (221 de 518, 42.66%) y demostrando circulación al menos desde el año 2004. Los datos compilados sugieren que PCV-3 es aparentemente capaz de causar una infección persistente, ya que 5 de 10 jabalíes capturados/re-capturados positivos a PCV-3 mostraron positividad en muestreos separados por más de 5 meses. La frecuencia de detección del genoma de PCV-3 también fue investigada por primera vez en diferentes muestras de tejido y heces. Se detectó el genoma de PCV-3 en todos los tipos de tejido analizados, siendo el linfonodo submandibular, tonsila, pulmón, hígado, bazo y riñón los órganos con mayor frecuencia de positividad. La cantidad de ADN en todas las muestras de PCR positivas para PCV-3 analizadas fue de moderada a baja. Todas las secuencias parciales y completas de PCV-3 obtenidas de jabalíes mostraron una elevada similitud nucleotídica (> 98%).

En conclusión, los resultados obtenidos en esta Tesis proporcionan datos relevantes sobre la epidemiología de este nuevo virus, PCV-3, tanto en

cerdos domésticos como en jabalíes. Además, la información filogenética sugiere una baja variabilidad genética de PCV-3, en contraste con otros virus de ADN monocatenario.

PUBLICATIONS

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

- Klaumann F., Franzo G., Sohrmann M., Correa-Fiz F., Drigo M., Núñez J.I., Sibila M., Segalés, J. Retrospective detection of Porcine *circovirus 3* (PCV-3) in pig serum samples from Spain. Transboundary and Emerging Diseases. 2018. 00:1–7. https://doi.org/10.1111/tbed.12876

- Klaumann F., Sibila M., Núñez J.I., Correa-Fiz F., Segalés J. Infection dynamics of *Porcine circovirus 3* in longitudinally sampled pigs from four Spanish farms. Submitted for publication.

- Klaumann F., Dias-Alves A., Cabezón O., Mentaberre, G., Castillo-Contreras R., López-Bejár M., Casas-Díaz E., Sibila M., Correa-Fiz F., Segalés J. *Porcine circovirus 3* is highly prevalent in serum and tissues and may persistently infect wild boar (Sus scrofa scrofa). Transboundary and Emerging Diseases. 2018. 00:1–11. https://doi.org/10.1111/tbed.12988

CHAPTER 1

INTRODUCTION

1.1. EMERGING INFECTIOUS DISEASES AND INFECTIONS

Newly emerging or re-emerging pathogens have threatened the survival of humans and animals for centuries. Historically, some emerging diseases have been responsible for widespread deadly outbreaks such as the Black Death pandemic in the 14th-century until the 18th century in Europe, which increased to 50% the mortality of the European population (25–40 million deaths) (Benedictow, 2006). Also, the 1918 influenza pandemic caused by a H1N1 strain resulted in approximately 50 million deaths (Taubenberger and Morens, 2006), and the more recently HIV/AIDS-related illnesses have caused around 35 million deaths so far (https://www.avert.org/global-hiv-and-aids-statistics).

An interesting study revealed that most of emerging pathogens are viruses (Taylor et al., 2001), suggesting a faster evolution than other pathogens. The evolution of emerging diseases is associated with some factors embedded in the concept "host-agent-environment triangle" (Davies, 2012). To infect the host and cause disease, the pathogen needs to evade host defenses, which may occur through single point mutations, genome rearrangements, recombination and/or translocation (Witzany, 2006). Genetic uniformity generated through genetic selection (Edfors-Lilja et al., 1998) and the fact that demographic changes, intensification of farming and international commerce have occurred intensively over the last decades, must be also considered as essential factors for the development of an emerging disease (Conway and Roper, 2000; Holmes and Rambaut, 2004; Woolhouse et al., 2001).

At least a dozen new diseases have been identified in the last decade, and traditional diseases that were thought to be in progress to eradication are resurging. Globally, infectious diseases remain the leading cause of death in humans. In 2012, the Middle East Respiratory Syndrome (MERS), caused by MERS-coronavirus (MERS-CoV), first emerged in the Kingdom of Saudi

Arabia causing so far 791 deaths (http://www.who.int/emergencies/mers-cov/en/). Another example is *Zaire ebolavirus*, the etiological agent of Ebola virus disease (EVD), probably the most deadly disease nowadays; just in 2014, EVD caused 28,616 infections with 11,310 deaths (http://www.who.int/csr/disease/ebola/en/). A year after, a new virus was discovered, able to infect fetuses and cause microcephaly: Zika virus (ZIKV) (Sarno et al., 2016). Infections with ZIKV have been reported in many countries all over the world, but mainly in South America (http://www.who.int/csr/disease/zika-virus/en/). Besides public health issues, emerging and reemerging diseases have a significant effect on socio-economy stability of societies whereas the disease are correlated with deaths, interference with travel, business and life activities (Morens and Fauci, 2013; Morse, 2012; Morse et al., 2012). Remarkably, most of the emerging diseases (60 to 80%) are caused by pathogens originated from animals (Karesh et al., 2012; Morens et al., 2004; Meslin et al., 2000), which emphasizes the importance on the "one health" concept. Created in 2004, the "one health" approach constitutes a global strategy for the integration of human, animal and environmental health regarding prevention and control of diseases, especially zoonosis (Day, 2011; Gibbs, 2014; Lerner and Berg, 2015).

As well as in humans, emerging diseases drastically affect animal populations, especially in food-producing animals. Livestock production in large communities (i.e., pig farms or poultry flocks) represents an excellent environment to facilitate the transmission and maintenance of viral infections within a population, contributing to the acquisition of pathogen genome modifications (mutation, recombination and reassortment) (La Rosa et al., 2012; Nichol et al., 2000). The intensification of livestock during the last four decades has probably been one of the main factors that contributed both to the

emergence of new pathogens and/or pathogen variants, leading to changes in the epidemiology and presentation of diseases (Fournié et al., 2015).

The number of viral infectious diseases in swine has significantly increased in the last 30 years. Several important viruses have been reported over the years, including *Porcine reproductive and respiratory syndrome virus* (PRRSV, family *Arteriviridae*), *Porcine circovirus 2* (PCV-2, family *Circoviridae*) and *Porcine epidemic diarrhea virus* (PEDV, family *Coronaviridae*). All of these caused a huge economic impact on the swine industry (Meng, 2012), reducing output, increasing the production costs and hence reducing product price. In the specific case of PRRSV infection, the disease is highly transmissible and can persist for long periods both in chronically infected animals and in the environment (Perez et al., 2015). PEDV can infect pigs of all ages; however ,the severity of the disease is observed mainly at early ages (Shibata et al., 2000), with reports indicating a mortality rate of 80%-100% (Sun et al., 2012). PCV-2 associated diseases have been linked with significant increase of postweaning mortality rates as well as negative impact on the growth of the animals (Hassing et al., 2006; Nielsen et al., 2008).

Besides those worldwide spread viruses, an important number of novel swine pathogens causing different types of diseases have been described (Canning et al., 2016; Wang et al., 2014). Although its economic impact might be variable, they are considered significant infection agents and their monitoring is already performed in some parts of the world. Some of these examples are *Porcine deltacoronavirus* (associated with diarrhea) (Wang et al., 2014), *Senecavirus A* (causing a vesicular disease and increased pre-weaning mortality) (Canning et al., 2016), *Porcine sapelovirus* (found in cases of polioencephalomyelitis) (Lan et al., 2011), *Porcine orthoreovirus* (assumed to cause diarrhea) (Narayanappa et al., 2015), *Atypical porcine pestivirus*

(cause of congenital tremors type II) (Postel et al., 2016) and HKU2-related coronavirus of bat origin (associated with a fatal swine acute diarrhoea syndrome) (Pan et al., 2017), among others.

Besides overt emerging diseases of swine, many other novel infectious agents have been detected in both healthy and diseased animals, and the real importance of them is under discussion. This group of agents is mainly represented by *Torque teno sus viruses* (TTSuV), *Porcine bocavirus* (PBoV), *Porcine torovirus, Porcine kobuvirus* (PKBV) and *Porcine sapelovirus* (PSV), which are thought to cause a subclinical infection with no defined impact on production (Meng, 2012; Song et al., 2014). An exception may be represented by *Hepatitis E virus* (HEV), since it seems fairly innocuous for pigs, but is considered an important zoonotic agent (Christensen et al., 2008; Liang et al., 2014). Very recently, in 2016, a novel member of the *Circoviridae* family named *Porcine circovirus 3* (PCV-3), with unknown effects on pigs, was discovered (Palinski et al., 2017; Phan et al., 2017).

The number of emerging diseases increased over last years. The 'newly' discovered pathogens oftentimes are present many years before the first detection, such as the case of *anelloviruses*. The first *anellovirus*, named *Torque teno virus*, was described for the first time in humans in 1997 (Nishizawa et al., 1997); however the presence of viral DNA was retrospectively found in a soldier belonging to Napoleon's Great Army, around 200 years ago (Bédarida et al., 2011). On the animal side, the case of the PCV-2 is interesting. This virus was discovered because of its association with severe clinical problems during mid-1990s (Clark, 1996; Harding, 1996), but retrospective studies showed its circulation, at least, since 1962 with no evidence of disease association (Jacobsen et al., 2009).

Most of the knowledge acquired on viral agents come from *in vitro* studies based on the gold-standard method for viral isolation, i.e. the

propagation of the virus in cell culture (Hsiung, 1984). However, some viruses cannot be propagated in the cellular platforms, which is a limiting factor to increase their knowledge on multiple aspects (disease reproduction, pathogenesis, immunity, etc.). In the last decades, a number of viral discoveries occurred due to the advance and development of technologies, especially sequencing. Initially, to sequence a pathogen genome (Sanger sequencing), or part of it, was dependent on previous knowledge of at least part of the genetic material of the pathogen (Sanger et al., 1977). But the advent on new technologies (metagenomics) circumvents both the unculturability and the requirement on previous knowledge on the viral genome (Eckburg et al., 2005; Mason et al., 2014). This latter group of techniques (next generation sequencing, NGS) allow analyzing the genomic sequences of a completely unknown organism and accurately characterizing the genetic composition of the individual (Wood and Salzberg, 2014). This novel technology has been used in many fields, including human, environment, plant and animal health research (Cárdenas et al., 2016; Casas and Maloy, 2014; Lepage et al., 2013; Roossinck et al., 2015). In the specific field of swine pathogen investigations, this metagenomics approach has been used to find unknown viruses such *Porcine bocavirus* (Blomström et al., 2009), *Atypical porcine pestivirus* (Hause et al., 2015) and, more recently, the above mentioned member of the *Circoviridae* family named PCV-3 (Palinski et al., 2017; Phan et al., 2016).

1.2. CIRCOVIRUSES

1.2.1. Taxonomy and classification

Circoviruses are small single-stranded DNA (ssDNA) viruses belonging to the family *Circoviridae* (Tischer et al., 1982). Six more families are classified as ssDNA virus according to the International Committee on

Taxonomy of Virus (ICTV): *Anelloviridae, Geminiviridae, Inoviridae, Microviridae, Nanoviridae and Parvoviridae.* These ssDNA viruses tend to be species-specific and infect a wide range of hosts, including vertebrates, invertebrates, bacteria or plants. All of them contain circular genomes with the exception of the linear genomes from the *Parvoviridae* family (ICTV, 2017-https://talk.ictvonline.org/).

Until 2016, the *Circoviridae* family was divided into two different genera: *Circovirus* and *Gyrovirus* (Pringle, 1999). A new taxonomical grouping has been recently established by the ICTV on the basis of the viral structure and genome; the genus *Gyrovirus* has been removed from the family *Circoviridae* and reassigned into the *Anelloviridae* family. In addition, the new taxon *Cyclovirus* has been included into the *Circoviridae* family (Rosario et al., 2017). This new genus is closely related with that of *Circovirus* members, with differences in the genomic structure such as the orientation of the major open reading frames (ORFs). Moreover, virions belonging to the genus *Cyclovirus* have been reported in both vertebrates and invertebrates, including both humans and other mammals (Ge et al., 2011; Phan et al., 2015; Sato et al., 2015; Smits et al., 2013; Zhang et al., 2014), birds (Li et al., 2010), and insects (Dayaram et al., 2013). Amazingly, none cyclovirus has been yet isolated in cell culture. In contrast, members of the *Circovirus* genus have been detected so far exclusively in vertebrates (Lukert et al., 1995). The first circovirus (*Psittacine beak and feather disease virus*) was described in avian species (Ritchie et al., 1989) and, subsequently, several reports revealed the presence of similar virions in swine (Todd, 2000), fishes (Lőrincz et al., 2012), bats (Li et al., 2010; Lima et al., 2015; Wu et al., 2012), chimpanzees (Li et al., 2010), dogs (Li et al., 2013) humans (Li et al., 2010) and minks (Lian et al., 2014).

Until 2016, just two species were known to be able to infect pigs: *Porcine circovirus 1* (PCV-1) and PCV-2. Thereafter, a new species was

discovered and the name PCV-3 was proposed (Palinski et al., 2017; Phan et al., 2016). However, due to its recent discovery, PCV-3 is not yet included in the last report of the ICTV. All species from *Cyclovirus* and *Circovirus* genera are displayed in the Figure 1-1.

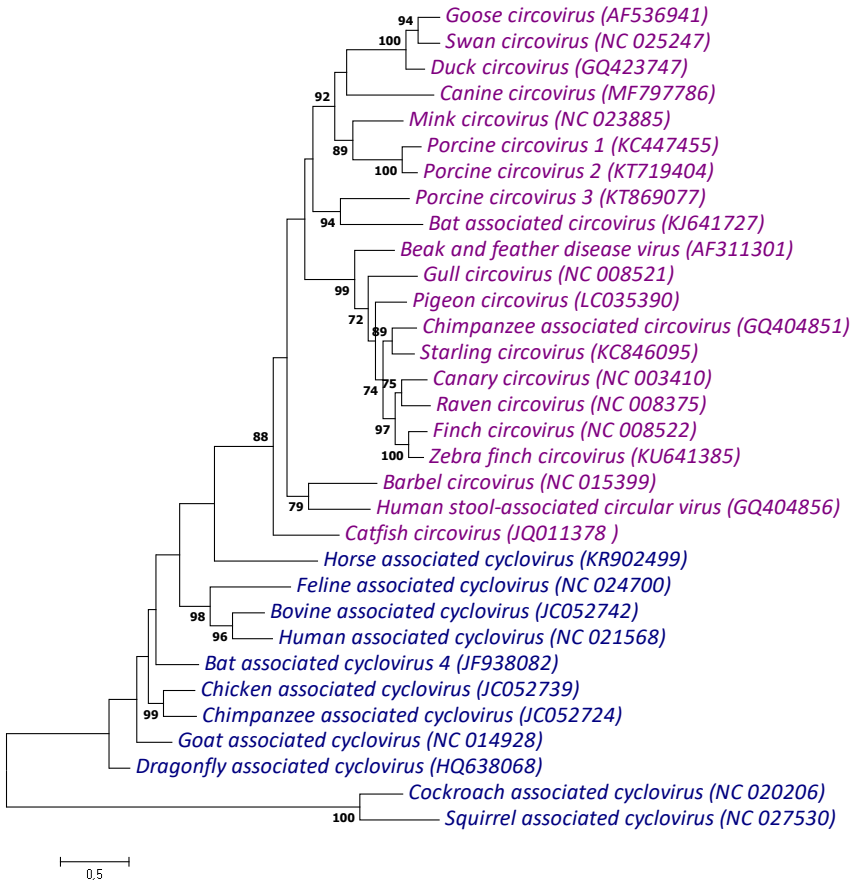

Figure 1-1. Phylogenetic tree based on the complete genome of one member of each *Cyclovirus* and *Circovirus* species. The tree was constructed by the Maximum-Likelihood method using the best-fit model K2G +G+I with 1,000 bootstrap replicates. Blue and purple colors represent the genus *Cyclovirus* and *Circovirus*, respectively.

1.2.2. Morphology of porcine circoviruses (PCVs)

Virions belonging to the *Circovirus* genus have a non-enveloped, icosahedral symmetry. They are constituted by 60 capsid protein subunits organized in a dodecahedral pentameter clustered unit (Crowther et al., 2003), with a diameter ranging from 13 to 25 nm. PCV-1 has a genome size ranging from 1,758 to 1,760 nucleotides (nt) (Fenaux et al., 2002, 2000a; Meehan et al., 1997), while the circular genomes of PCV-2 and PCV-3 consist of 1,766-1,769 and 1,999 to 2,001 nt, respectively (Table 1-1) (Fenaux et al., 2000a; Fux et al., 2018; Guo et al., 2010; Palinski et al., 2017; Zhang et al., 2015). The genomic organization for PCVs is similar with three major open reading frames (ORFs) arranged in the strands of the replicative form (RF) (Palinski et al., 2017). The RF is the double-stranded DNA (dsDNA) formed during rolling circle replication (RCR). For PCV-1, a total of six ORFs larger than 200 nt (Mankertz et al., 1997; Tischer et al., 1995b) or seven ORFs capable to encode proteins larger than 5kDa, have been predicted on both DNA strands (Meehan et al. 1997). In addition, PCV-2 contains eight more predicted ORFs, but just ORF4 has been characterized in more detail (Gao et al., 2014; He et al., 2013; Lin et al., 2018).

ORF1 is located on the positive strand and considered the most conserved region of the circovirus genome (Mankertz et al., 2004). The origin of replication (*ori*), constituted by a conserved nonanucleotide motif [(T/n)A(G/t)TATTAC], is located on the same strand as ORF1 and, consequently, this frame is involved in rolling circle replication (Rosario et al., 2012). ORF1 encodes for Rep and Rep' proteins involved in replication initiation, of 312 aa and 168 aa, respectively, in PCV-1, and of 314 aa and 297 aa, respectively, for PCV-2 (Hamel et al., 1998). ORF1 apparently codes for a single replicase protein in PCV-3, of 296-297 aa (Palinski et al., 2017; Phan et al., 2016).

ORF2 encodes the only structural protein which composes the capsid of the virus (Cap protein). It consists of 230-233 aa for PCV-1, 233-236 aa for PCV-2 (Hamel et al., 1998; Huang et al., 2011; Lefebvre et al., 2009) and 214 aa for PCV-3 (Palinski et al., 2017; Phan et al., 2016). ORF2 is located on the negative DNA viral strand and *cap* protein is considered the most variable viral protein (Fenaux et al., 2000b; Grierson et al., 2004; Knell et al., 2005), as well as the most immunogenic (Nawagitgul et al., 2002). Phylogenetic studies revealed a nucleotide similarity of 67% in *cap* protein between PCV-1 and PCV-2 (Mankertz et al., 1998); moreover, the similarity in this protein is much lower (24%) among PCV-1 and PCV-3 (Phan et al., 2016) while being 26-37% between PCV-2 and PCV-3 (Palinski et al., 2017; Phan et al., 2016).

The ORF3 codifies for a non-structural protein with apoptotic capacity (Hamel et al., 1998; Huang et al., 2013); the gene is oriented in the opposite direction of ORF1, also in the negative strand. ORF3 protein consists of 206 aa for PCV-1, 104 aa for PCV-2 and 231 aa for PCV-3 (Liu et al., 2005; Palinski et al., 2017). The apoptotic activity, for both PCV-1 and PCV-2, has been described both under *in vitro* and *in vivo* conditions (Karuppannan and Kwang, 2011; Lin et al., 2011), while its function in PCV-3 is still unknown.

Lastly, ORF4, also located in the negative strand, has only been described in the PCV-2 genome. This gene codifies for a protein with anti-apoptotic function, of approximately 60 aa (Gao et al., 2014; He et al., 2013).

Table 1-1. Description of the three major ORFs in PCV-1, PCV-2 and PCV-3.

Porcine circovirus	**Size (nt)**	**ORF1**		**ORF2**		**ORF3**	
		Protein	Size (aa)	Protein	Size (aa)	Protein	Size (aa)
PCV-1	1,758-1,760	*Rep* *Rep'*	312 168	*Cap*	230-233	NS	206
PCV-2	1,766-1,769	*Rep* *Rep'*	314 297	*Cap*	233-236	NS	104
PCV-3	1,999-2,001	*Rep*	296-297	*Cap*	214	Unknown	231

NS: Non- structural protein; nt: nucleotides; aa: amino acids

1.3. *PORCINE CIRCOVIRUS 1* (PCV-1)

1.3.1. History

By mid 1970s, a small and spherical non-cytopathic virus was found persistently in the porcine kidney cell-line (PK-15, ATCC CCL-33). This virus, suspected to have an RNA genome, was initially classified as a picornavirus-like agent (Tischer et al., 1974). The origin of this viral contamination in the PK-15 cell lines was unknown, but it was speculated that its introduction occurred from contaminated serum used in the growth medium or came from the original swine tissue (Dulac and Afshar, 1989).

Few years after, such PK-15 cell contaminant virus was confirmed to have a closed circular ssDNA genome with the virion measuring about 17 nm in diameter; the name *Porcine circovirus* (PCV) was proposed (Tischer et al., 1982). Further studies elucidated that its DNA replication occurred in actively dividing cells, depending on cellular enzymes expressed during the S phase of

cell growth (Tischer et al., 1987). Besides *in vitro* work, the virus was also tested in pigs, concluding that swine herds usually had specific anti-PCV antibodies, although no association with disease was found (Allan et al., 1995; Dulac and Afshar, 1989; Tischer et al., 1995b).

Experimental studies also confirmed that PCV-1 was no pathogenic to pigs since the inoculation in minipigs implied antibody development, but no disease was observed (Tischer et al., 1986). Similar findings were obtained by means of an experimental infection of colostrum deprived domestic pigs (Allan et al., 1995). PCV-1 is now known to be able to replicate not only in PK-15, but also in Vero cells (Allan et al., 1994a; Tischer et al., 1982). PCV-1 (PCV at that time) was then classified by the ICTV as a member of the family *Circoviridae* (Lukert *et al*., 1995).

1.3.2. Epidemiology

The PCV-1 was initially found in Germany, but subsequent reports demonstrated a worldwide distribution since the virus was detected in North America (Dulac and Afshar, 1989), Europe (Allan et al., 1994b; Tischer et al., 1982) and Oceania (Muhling et al., 2006).

PCV-1 antibody prevalence in domestic pig has been reported to be variable, but in some cases reaching 100% (Labarque et al., 2000), depending on age. It has been suggested that the time at which PCV-1 infection occurs is mainly during the nursery phase, with a decreasing rate of viral detection afterwards, and an increasing antibody prevalence during the postweaning period (Tischer et al., 1995a).

1.3.3. Disease association

Porcine circovirus 1 is considered non-pathogenic to pigs based on experimental infections and its wide serological distribution in absence of

evident disease (Allan et al., 1995; Beach et al., 2010; Finsterbusch and Mankertz, 2009; Tischer et al., 1986). However, the PCV-1 genome has been found in piglets with congenital tremors type AII and in stillborns, suggesting a possible vertical transmission (Allan et al., 1995; Choi et al., 2002; Stevenson et al., 2001). Likewise, as the virus has been detected in the milk of sows, the possibility of transmission via colostrum to the newborn was proposed (Shibata et al., 2006). Interestingly, when the first initial case of PCV-2-systemic disease (called postweaning multisystemic wasting syndrome at that time) was firstly described in France, the very initial case detected PCV-1 together with PCV-2 (LeCann *et al*., 1997).

The experimental inoculation of PCV-1 prompted its detection at different time points, but once again, no clinical disease or postmortem lesions were observed (Allan et al., 1995; Fenaux et al., 2003; Krakowka et al., 2000; Tischer et al., 1986). The virus has been detected in both sera and plasma samples, and in different tissues including nasal mucosa, lymph nodes, intestine, liver and, predominantly, in lung, spleen and thymus (Allan et al., 1995). The distribution of PCV-1 into different tissues seems to occur via blood after the infection of monocytes (Allan et al., 1995); however, the primary site to its replication is still unknown.

1.3.4 Virus/antibody detection methods

Since this infectious agent is not considered of diagnostic interest for veterinarians, minimal laboratory developments occurred over time. In consequence, and mainly for research purposes, antibodies are usually detected by means of immunoperoxidase monolayer assay on PK-15 cells (Han et al., 2016) and genome by different PCR techniques (Huang et al., 2004; Kumar et al., 2012; Quintana et al., 2006). In early times, an ELISA was also developed (Tischer et al., 1995b), but only used for research purposes. PCV-1 can also be

cultured and propagated cell lines, such as PK-15 or ST cells (Tischer et al., 1982).PCV-1 can be also detected by *in situ hybridization* (Nawagitgul et al., 2000).

1.4. *PORCINE CIRCOVIRUS 2* (PCV-2)

1.4.1. History

An apparently new disease called postweaning multisystemic wasting syndrome (PMWS) was diagnosed in 1991 in Western Canada (Clark, 1997; Harding, 1996). The disease, detected in a single swine herd, was characterized by wasting, jaundice and respiratory signs such as dyspnea and tachypnea (Clark, 1997; Harding, 1996). Gross and microscopic lesions including interstitial pneumonia, lymphadenopathy, lymphocytic granulomatous hepatitis and nephritis were described in affected cases (Rosell et al., 1999). *In situ* hybridization, immunohistochemistry, electron microscopy and virus isolation helped to elucidate the presence of a DNA virus in these lesions, with similar structure to the existing PCV (afterwards named PCV-1) (Allan et al., 1998).

Phylogenetic analyses demonstrated differences between the already existing PCV and the virus found in these PMWS-affected animals. In consequence, a new nomenclature for these viruses was proposed: PCV-1 for the non-pathogenic virus and PCV-2 for the virus found in animals affected by PMWS (Meehan et al., 1998). The disease was considered devastating for more than a decade in different countries, causing significant economic losses in the swine industry (Segalés et al., 2013). Since its discovery, several efforts were made to develop a vaccine against this devastating disease (Afghah et al., 2017; Karuppannan and Opriessnig, 2017). Nowadays, the disease is under

control by means of vaccination , being currently the most used pig vaccine worldwide (Segalés, 2015). Besides PMWS, currently known as PCV-2-systemic disease (PCV-2-SD), the virus is involved in several conditions collectively designated as porcine circovirus diseases (PCVD) (Segalés et al., 2005a).

Initial sequencing studies concluded that all PCV-2 strains were fairly similar with nucleotide sequence identity higher than 93%. However, phylogenetic studies indicated the existence of at least two genetic groups (Mankertz et al., 2000), which were subsequently named as genotypes (Segalés et al., 2008). To date, six different genotypes (PCV-2a to PCV-2f) have been defined for PCV-2 based on the construction of p-distance/frequency histograms and establishing cut-off values to distinguish among them (Bao et al., 2017; Davies et al., 2016; Franzo et al., 2015b; Guo et al., 2010; Segalés et al., 2008). PCV-2a, PCV-2b and PCV-2d are the most prevalent genotypes in the pig population (Franzo et al., 2015b; Olvera et al., 2004; Wang et al., 2013; Wiederkehr et al., 2009). Until the beginning of the year 2000, the most frequent genotype found was PCV-2a. Nowadays PCV-2b is the most common genotype in the pig population (Allan et al., 2012; Segalés et al., 2013) but PCV-2d has shown an increasing prevalence around the world lately (Franzo et al., 2015b; Guo et al., 2010; Xiao et al., 2015).

1.4.2. Epidemiology

After the first report in North America, PCV-2 was found in all continents indicating a widespread distribution. Retrospective studies demonstrated that the virus was present in Europe at least since 1962 with a low prevalence in the first tested years and an increase in frequency after 1985 (Jacobsen et al., 2009).

The virus is detected mainly in domestic pigs, but wild boar, feral pigs and peccaries are also susceptible to PCV-2 infection (Ellis et al., 2003; Franzo et al., 2015a; de Castro et al., 2014; Schulze et al., 2004). PCV-2 is considered a virus infecting solely members of the *Suidae* family, but it has been sporadically found in other species. The virus was detected in cattle by PCR in cases of bovine respiratory disease and abortion (Nayar et al., 1999), and from a bovine hemorrhagic diathesis disease (Kappe et al., 2010); however, the pathogenesis and the importance of PCV-2 in ruminants and other species are still unknown since no clinical disorders or lesions, as well as viremia and antibody response were observed after PCV-2 *in vivo* infection (Allan et al., 2000; Ellis et al., 2001; Quintana et al., 2002). PCV-2 has also been found in different tissues from dead mice and rats collected outside pig farms, suggesting that these rodents carry the virus and may act as reservoirs and vectors for PCV-2 (Lőrincz et al., 2010). In fact, experimental inoculation in mice demonstrated the ability of the virus to replicate in this species. The virus has also been detected in *Musca domesticus*; in this particular study, PCV-2 sequences were identical with those of pigs, suggesting the fly as a potential in-farm vector of PCV-2 (Blunt et al., 2011). Anyway, with the potential exception of mice (some experiments suggest viral replication) (Cságola et al., 2008; Kiupel et al., 2001), it has not been yet demonstrated that PCV-2 truly infects non-suidae species.

Transmission of this virus mainly occurs by direct contact with an infected host (Grau-Roma et al., 2008; Rose et al., 2012; Segalés et al., 2005b). *Porcine circovirus 2* can be found in the nasal cavity to fairly high loads, but its genome has also been detected in a variety of samples such as serum, oral fluids, stool, saliva, urine, colostrum, milk, semen, bronchial and ocular secretions as well as in a myriad of tissues (Ha et al., 2009; Krakowka et al., 2000; Larochelle et al., 2000; Patterson and Opriessnig, 2010; Rose et al.,

2012; Sibila et al., 2004; Rosell et al., 1999). Indirect route of transmission is also possible through aerosols, contaminated inanimate objects or with living vectors (Madson and Opriessnig, 2011; Verreault et al., 2010). Moreover, vertical transmission has been also reported since PCV-2 can be detected in embryos and fetuses (Bielanski et al., 2004; O'Connor et al., 2001; Rose et al., 2012).

1.4.3. Disease association

In the post-natal infection, upon experimental inoculation, the primary sites of virus replication are tonsils and lymphoid organs around this region, as well as mesenteric lymph node and Peyer's patches (McNair *et al*., 2007; Rosell *et al*., 1999). The virus can be detected in blood and tissues, and viral load increases between 14 and 21 dpi (Meerts et al., 2005; Resendes et al., 2011; Rovira et al., 2002). In those animals developing disease, PCV-2 is associated with depletion of lymphocytes (Allan et al., 1998; Rosell et al., 1999; Sanchez et al., 2003; Yu et al., 2007) and disruption (Vincent et al., 2005) or decrease of cellular proliferation (Mandrioli et al., 2004).

As indicated above, the terminology PCVD includes all these clinical and subclinical presentations of PCV-2 infection. The most economically important conditions are both PCV-2-SD and PCV-2-subclinical infection (PCV-2-SI). This latter one was discovered by means of PCV-2 vaccine use (Segalés, 2015). PCV-2 is also associated to other clinical expressions like PCV-2 reproductive disease (PCV-2-RD) (Brunborg et al., 2007; West et al., 1999) and porcine dermatitis and nephropathy syndrome (PDNS) (Drolet et al., 1999; Segalés et al., 1998). PCV-2 lung disease (PCV-2-LD) and PCV-2 enteric disease (PCV-2-ED) have also been described in the literature, but recent studies suggest that are part of the PCV-2-SD scenario rather than independent conditions (Baró et al., 2015; Ticó et al., 2013).

Currently, PCV-2-SI is the most common form of PCVD, and is characterized by a decreased average daily weight gain with no overt clinical signs or lesions (Segalés, 2012). Such subclinical scenario is the one that prompted the widespread use of PCV-2 vaccines, since their positive effect was evident in both clinically and subclinically affected farms (Segalés, 2015). Even the PCV-2-SI is under control by vaccination, the infection seems not able to be eradicated from the pig population (Feng et al., 2014).

On the other hand, PCV-2-SD is characterized by wasting, weight loss, pallor of the skin, respiratory distress, diarrhea and icterus. The disease usually appears between 7 and 16 weeks of age and the morbidity and lethality range from 4 to 30% and 70-80%, respectively (Darwich et al., 2004; Segalés and Domingo, 2002). PCV-2 is essential for the development of PCV-2-SD; however, some co-factors have been associated with the clinical presentation. Co-infections with PRRSV, *Porcine parvovirus* (PPV), *Mycoplasma hyopneumoniae* and other pathogens can be found frequently with PCV-2 under field conditions (Opriessnig and Halbur, 2012). Moreover, the experimental co-infection of PCV-2 with some of these infectious agents facilitated the reproduction of PCV-2-SD (Allan et al., 1998; Rovira et al., 2002), further emphasizing the multifactorial nature of the disease. Other factors such as immunomodulation, viral and host factors, and management (hygiene, biosecurity, nutrition and vaccination programs) are also considered to exert significant effects on PCV-2-SD severity and impact (Gillespie et al., 2009; Grau-Roma et al., 2011; Rose et al., 2012). From a pathological point of view, lymphadenopathy and lung lesions are common, but lesion in liver, kidneys, stomach and intestines may be detected (McNair et al., 2007). Microscopically, lymphocyte depletion with infiltration of histiocytic and/or multinucleated giant cells is the hallmark lesion which really defines the disease (Clark, 1997; Rosell et al., 1999); other findings such as lympho-

histiocytic inflammation can be found virtually in all organs of pigs severely affected by PCV-2-SD (Clark, 1997; Segalés et al., 2004).

In addition, PCV-2-RD was firstly described in aborted fetuses with a severe diffuse myocarditis (West et al., 1999); subsequent reports showed the presence of PCV-2 in stillborn and also aborted and/or mummified fetuses (Brunborg et al., 2007; O'Connor et al., 2001). Experimental infection in different time-points of the gestation revealed highly susceptibility of virus replication in mid-gestation fetuses, but a decreasing susceptibility onwards (Sanchez et al., 2004, Sanchez et al., 2003, Sanchez et al., 2001). The clinical picture in sows and fetuses affected by PCV-2-RD depends on the gestational phase, the immune response and timing of infection. Based on *in vitro* studies, an early infection (1-35 days of gestation) would result in embryonic death, and in consequence, in return-to-estrus, pseudo-pregnancy or small litter sizes (Mateusen et al., 2007). When PCV-2 infection occurs between 35 and 70 days of gestation, mummified fetuses and abortion would be the major outcome observed in the herd. Infection after 70 days is not expected to cause clinical evident problems, although an increase in the rate of stillborn or weak-born piglets may be observed, as well as delayed farrowing (Oropeza-Moe et al., 2017; Sanchez et al., 2004, Sanchez et al., 2003, Sanchez et al., 2001). In fetuses, the heart is the main target organ for PCV-2 replication (Sanchez et al., 2001;Madson et al., 2009), and fibrosing and/or necrotizing myocarditis can be seen (West et al., 1999). Other gross lesions observed in aborted fetuses are pneumonia, hepatomegaly, hydrothorax, ascites and subcutaneous edema (O'Connor et al., 2001). High virus load can be found in the heart, although the virus is detectable as well in spleen, lymph nodes, lung and liver (Sanchez et al., 2003, Sanchez et al., 2001).

PDNS is clinically characterized by red-to-purple macules and papules in the skin, usually on the hind limbs and perianal area, but in some cases the

lesions can be observed in all body as apparent scars (Drolet *et al*., 1999). Microscopically, hemorrhagic and necrotic skin associated with necrotizing vasculitis are observed and/or enlargement of kidneys with generalized cortical petechiae (Segalés et al., 1998; Segalés et al., 2004). The disease normally affects fatteners, and occasionally nursery pigs and sows, but when animals are severely affected, they die few days after the onset of clinical signs. The frequency of PDNS is usually lower than 1%, although the mortality rate can reach up to 50-100% (Segalés et al., 2004, 1998). PDNS is considered an immunocomplex disease in which PCV-2 has been postulated as the potentially related antigen (Segalés et al., 2012). However, the experimental evidence of PCV-2 as the trigger antigen for PDNS has been never demonstrated.

1.4.4. Virus/antibody detection methods and disease diagnoses

Several laboratory techniques have been developed in the last 20 years to detect PCV-2 genome, antigen or its antibodies. Besides the use of histopathology to detect lesions, most used techniques to detect the virus in diagnostic approaches include PCR (usually quantitative methods), immunohistochemistry (IHC) and *in situ* hybridization (ISH) (Brunborg et al., 2007; Olvera et al., 2004; Rosell et al., 1999). The latter two techniques are especially useful since they allow the presence of virus together with the histological lesions with presence of the virus. PCV-2 antibody detection does not constitute diagnosis of PCVDs, but ELISA techniques nowadays and immunoperoxidase monolayer assay (IPMA) or immunofluorescent assay (IFA) formerly, are excellent techniques to monitor viral infection and/or vaccination (Fablet et al., 2017; Feng et al., 2014; Shin et al., 2015; Sun et al., 2010).

Through quantitative PCR methods (qPCR) is possible to quantify the amount of PCV-2 in different samples of alive or death pigs. Although many authors proposed a specific PCV-2 threshold associated with clinical disease, a final consensus has not been achieved, since sensitivity and specificity depends on the particular laboratory technique (Harding et al., 2008; Hjulsager et al., 2009). Anyway, viral loads in serum similar or higher than 10^7 viral genome/mL or mg. tend to be associated with PCVDs (Brunborg et al., 2007; Fort et al., 2007; Grau-Roma et al., 2009). Laboratory techniques must be combined with a clinical diagnostic criterion in order to establish the case definition of the disease. Table 1-2 summarizes the major diagnostic criteria for the abovementioned PCVDs.

Table 1-2. Summary of diagnostic criteria according to the clinical syndromes and subclinical PCV-2 infection (Adapted from Segalés, 2012).

PCVD	Diagnostic criteria
PCV-2-SI	• Absence of clinical signs • Viral detection (low amount of PCV-2) • Nor/minimal microscopic lesions
PCV-2-SD	• Presence of clinical signs compatible with the disease characterized by wasting and/or growth retardation • Presence of microscopic lesions compatible with PCVD, such as lymphocyte depletion with lymphohistiocytic to granulomatous inflammation of the lymphoid tissues • Virus detection in the lesions
PCV-2-RD	**<u>Late gestation</u>** • Reprodutive failure • Microscopic lesions in the heart of fetuses such as fibrous to necrotizing myocarditis • Virus detection (moderate to high amount of PCV-2 DNA) in the heart of fetuses **<u>Early gestation</u>** • Return-to-estrus • PCV-2 genome or antibodies around or following the return-to-estrus
PDNS	• Skin lesions characterized by hemorrhagic and necrotizing areas and/or swollen • Kidney lesions characterized macroscopically by pale kidneys with cortical petechia and microscopically by systemic necrotizing vasculitis and necrotizing and fibrinous glomerulonephritis

1.5. *PORCINE CIRCOVIRUS 3* (PCV-3)

1.5.1. History

Porcine circovirus 3 (PCV-3) was described in 2015 in North Carolina (USA). It was specifically detected in sows with a PDNS-like condition that also experienced an increase of 10.2% in the mortality rate and a decrease of 0.6% in the conception rate (Palinski et al., 2017). Moreover, several animals also had reproductive failure. Aborted fetuses and organs such as skin, kidneys, lungs and lymph nodes from sows were collected for further analyses. The histological results were consistent with PCV-2-SD; however, immunohistochemistry (IHC) and quantitative PCR (qPCR) methods to detect PCV-2 yielded negative results. Moreover, these samples were also negative for PRRSV and *Influenza A virus* (IAV). The NGS analyses on both, a tissue homogenate from three fetuses and from homogenized tissues from sows with PDNS-like lesions, revealed the presence of an uncharacterized virus (Palinski et al., 2017). Further studies using rolling circle amplification followed Sanger sequencing revealed a circular genome assembly of 2,000 nucleotides. Finally, three ORFs encoding for potential proteins of more than 200 aa were predicted. Two of these ORFs were oriented in opposite directions, which had similarity to ORF1 and ORF2 from other members of the genus *Circovirus* (Figure 2). Approximately 54% of the mapped reads demonstrated high similarity (98%) with the partial genome of a circovirus strain found in pork meat products named PORKNW2/USA/2009. Phylogenetic analyses suggested a closest relationship among the newly described PCV-3 and *Canine circovirus.* In addition, a brief retrospective study was performed by qPCR on serum samples from animals clinically affected by PDNS-like lesions (but negative for PCV-2 by IHC) and pigs with porcine respiratory diseases, showing PCV-3 qPCR

positivity in 93.75% and 12.5% of the analyzed samples, respectively (Palinski et al., 2017).

Curiously, also in the USA and almost concomitantly, a clinical picture pathologically characterized by multi-systemic and cardiac inflammation of unknown etiology was detected (Phan et al., 2016). Tissues from three affected pigs of different age (3 to 9 week-old animals) were analyzed by NGS methods. Such analyses resulted in the discovery of a novel viral sequence characterized by a circular genome containing 2,000 nucleotides. Further sequence analyses indicated the existence of three putative ORFs: ORF1, ORF2 and ORF3 (Phan et al., 2016). Besides NGS, *in situ* hybridization was performed in one out of these three pigs, confirming PCV-3 DNA in the myocardium, more specifically in myocardiocytes, leiomyocites of an inflamed arteriole and in inflammatory cells.

Based on these two initial works, the name PCV-3 was proposed as the third species of circoviruses that affect pigs, since pairwise analysis demonstrated significant divergence with the existing PCVs (Figure 1-2). Moreover, the novel sequences showed less than 70% of identity in the predicted whole genome and capsid protein aa sequence compared to the other members of the *Circovirus* genus (Phan et al., 2016).

Afterwards, a significant number of publications showed the presence of PCV-3 in pigs from different countries around the world and affected by several pathological conditions. Through retrospective studies the circulation of the virus was confirmed, at least since 1993 in Sweden (Ye et al., 2018) and 1996 in China (Sun et al., 2018). In order to understand the evolutionary history of PCV-3, a few phylogenetic studies were conducted on retrospective data suggesting that PCV-3 strains spread approximately in the middle of 1960s (Fu et al., 2017; Saraiva et al., 2018), as a result of the recombination with other circoviruses (Franzo et al., 2018e; Ku et al., 2017).

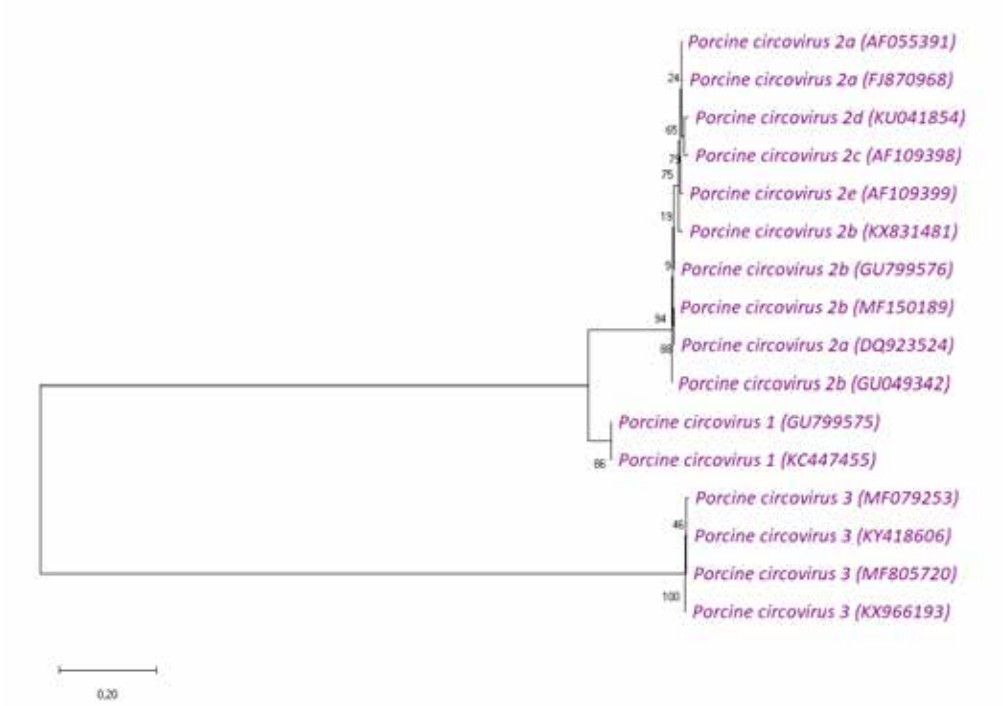

Figure 1-2. Phylogenetic tree based on the complete genome of some porcine circoviruses detected in different countries. The phylogenetic tree was constructed by the Maximum-Likelihood method using the best-fit model K2G +G with 1,000 bootstraps replicate.

A high identity between PCV-3 sequences (ranging from 96% to 100%), independently of the tested year and country was confirmed through sequencing and phylogenetic analysis (Fux et al., 2018; Ku et al., 2017; Zheng et al., 2017; Zou et al., 2018). In addition, two main groups classified as PCV-3a and PCV-3b and several sub-clusters were proposed (Fu et al., 2017; Fux et al., 2018; Li et al., 2018a), based on differences found in the aa sites 122 and 320 in both PCV-3a and PCV-3b (S122A and A320V). In fact, certain different antigenicity among groups has been proposed (Li et al., 2018a), although is still too early to discuss about potential different genotypes or subgroups for PCV-3.

1.5.2 Epidemiology

After the first descriptions in the USA, countries from Asia, Europe and South America (Figure 1-3) have reported the presence of PCV-3 genome in different types of samples in the domestic pig population (Faccini et al., 2017; Franzo et al., 2018b; Fu et al., 2017; Ku et al., 2017; Stadejek et al., 2017; Sun et al., 2018; Tochetto et al., 2017; Yuzhakov et al., 2018).

Figure 1-3. Countries in red are those that have been so far reported PCV-3 positive samples in domestic pig.

It is still too early to indicate the prevalence of infection with PCV-3, but the frequency of viral detection by PCR in pigs according to the collected samples around the world is displayed in Table 1-3.

PCV-3 DNA has been detected in pigs from all tested ages, from adults (sows) to mummified fetuses and stillborns. On one hand, a study performed in Poland indicated that the most frequently infected age-group comprised nursery and fattening pigs, being the highest prevalence in animals after five weeks of age (Stadejek et al., 2017). Also, PCV-3 was found in moderate to

high rate in sera pools from sows in Poland (Stadejek et al., 2017) and Thailand (Kedkovid et al., 2018a).

Besides domestic pigs, only one study has reported the detection of PCV-3 in wild boar in Europe. The viral DNA sequences retrieved from wild boars showed more than 98% of similarity with the available sequences from domestic pigs (Franzo et al., 2018d). The prevalence found in tested serum samples (33%) was similar or higher than that found in domestic pigs. Additionally, infection susceptibility was associated with the age; juvenile animals were statistically less often PCV-3 PCR positive than older ones wild boars (Franzo et al., 2018d). Accordingly to the obtained data, a potential reservoir role of the wild boar in respect PCV-3 infection was suggested. The viral DNA sequences retrieved from wild boar had more than 98% of similarity with the available sequences from domestic pigs (Franzo et al., 2018d).

PCV-3 genome has been detected by PCR in oral fluids and nasal swabs (Franzo et al., 2018b; Kwon et al., 2017) as well as in feces (Collins et al., 2017), semen (Ku et al., 2017) and colostrum (Kedkovid et al., 2018a). Kedkovid and collaborators (2018a) found a positive correlation between serum samples and colostrum, suggesting that the colostrum is influenced by the viremic stage. No specific studies have been performed on the detection of the virus in the environment, but one study indicates that the virus was found in 2 out of 4 sponge samples used on trucks after sanitation (Franzo et al., 2018a).

Table 1-3. Reports describing PCV-3 frequency of detection on different countries and sample types.

Reference	Country	Sample type	PCV-3 Positive (n)	Tested samples (n)	Frequency of detection (%)
Collins et al., 2017	Ireland	Tissue and feces	52	313	16.61
Fu et al., 2017	China	Tissue and stillborn	76	285	26.67
Kwon et al., 2017	South Korea	Oral fluid	159	360	44.17
Ku et al., 2017	China	Tissue, stillborn, semen and serum	77	222	34.68
Palinski et al., 2017	USA	Serum	47	150	31.33
Stadejek et al., 2017	Poland	Serum	55	215	25.58
Xu et al., 2017	China	Tissue and serum	53	170	31.18
Zhai et al., 2017	China	Tissue and serum	84	506	16.60
Zheng et al., 2017	China	Tissue	132	222	59.46
Wen et al., 2017	China	Tissue and serum	50	155	32.26

Table 1-3 (continuation). Reports describing PCV-3 frequency of detection on different countries and sample types.

Reference	Country	Sample type	PCV-3 Positive (n)	Tested samples (n)	Frequency of detection (%)
Franzo et al., 2018[a]	Italy	Sponge sample	2	4	50.00
Franzo et al., 2018b	Denmark	Tissue and serum	44	78	56.41
Franzo et al., 2018b	Italy	Tissue and serum	36	91	39.56
Franzo et al., 2018b	Spain	Serum (pools)	14	94	14.89
Hayashi et al., 2018	Japan	Tissue	7	73	9.59
Kedkovid et al., 2018[a]	Thailand	Colostrum	17	38	44.74
Kedkovid et al., 2018b	Thailand	Tissues and serum	33	103	32.04
Sun et al., 2018	China	Tissue	13	200	6.50
Zou et al., 2018	China	Serum	62	190	32.63
Zhao et al., 2018	China	Tissue	40	272	14.71
Ye et al., 2018	Sweden	Tissue	10	49	20.41

PCV-3 seems to be restricted to *Suidae* species, as the majority of the studies have been reported the presence of PCV-3 in domestic pigs. Just one study confirmed, the susceptibility of wild boars to PCV-3 as described above (Franzo et al., 2018d). However, there is also one single report were PCV-3 genome was detected in 4 out of 44 (9.09%) of the tested sera of dogs from China. The authors suggested that the virus might infect, therefore, non-porcine species (Zhang et al., 2017). To date, there is no evidence regarding susceptibility to PCV-3 infection in other species.

1.5.3. Disease association

PCV-3 genome has been found in tissues of pigs with several clinical/pathological conditions; however, the virus DNA has also been found in apparently healthy animals (Franzo et al., 2018b; Palinski et al., 2017; Zheng et al., 2017). Studies aimed to quantify the viral genome revealed variable DNA load in serum samples; (10^2-10^7 copies/mL) (Palinski et al., 2017) and tissues (10^4-10^{11} copies/mg) (Kedkovid et al., 2018b; Xu et al., 2018). The amount of DNA for stillborn or fetal tissues ranged from 10^6 to 10^9 copies/mg (Faccini et al., 2017; Palinski et al., 2017). When low viral loads are found, they are likely to be associated with a subclinical infection. For example, an association between high viral load and severity of disease, has been reported for PCV-2 in PCV-2-SD (Olvera et al., 2004) and PCV-2-RD contexts (Brunborg et al., 2007). However, the meaning of a given genome viral load in tissues or other samples for PCV-3 is still to be elucidated.

The clinical conditions in which PCV-3 has been tested and found is summarized in Table 1-4. However, it is worthy to state that the mere detection of viral genome does not imply that the virus is the cause of the observed condition. Therefore, a significant number of the still limited literature reports on PCV-3 fail regarding the establishment of causality of the clinical problem

by the virus. Thus, this section compiles the peer-reviewed papers that, while exploring certain disease scenarios, they found PCV-3 DNA. Noteworthy, in most of the cases, there are not complete diagnostic studies, but only the detection of the viral genome in a number of pigs affected by different clinical signs.

Regarding sows, PCV-3 has been found in animals with clinical signs compatible with PDNS in USA. In these affected farm, conception rates decreased and the sow mortality was above normal rates (Palinski et al., 2017). In China, PCV-3 was found in serum samples from sows with reproductive problems characterized by acute loss of neonatal piglets (Ku et al., 2017). Moreover, a comparative study between healthy sows and sows with a clinical picture characterized by prolonged reproductive failure (including increase in the abortion and sow mortality rates) revealed that PCV-3 positivity was higher in affected sows than in healthy ones (39 out of 84 diseased sows; 23 out of 105 healthy sows) (Zou et al., 2018). Viral genome has also been found in tissues from stillborn in farms experiencing reproductive failure in China (Ku et al., 2017; Zheng et al., 2017; Zou et al., 2018).

In addition, it is not uncommon to find PCV-3 DNA in pigs with respiratory disorders, as already indicated in the first report on this virus (Palinski et al., 2017). Two more studies reported PCV-3 genome in animals with abdominal breathing and pathological lesions described by lung swelling and congestion in China (Shen et al., 2017; Zhai et al., 2017). More recently, viral genome has been detected in fattening pigs from Thailand suffering from porcine respiratory disease complex (PRDC), characterized by cough, dyspnea, fever, anorexia; the prevalence was higher in diseased animals when compared to healthy ones (Kedkovid et al., 2018b).

One single study described PCV-3 in weaned pigs that suffered from gastro-intestinal disorders (diarrhea), showing higher prevalence in pigs with

severe clinical signs compared to those with moderate signs or no diarrhea (Zhai et al., 2017). *Porcine circovirus 3* was also detected by qPCR in different tissues from piglets with congenital tremors. Interestingly, PCV-3 was the only pathogen found in the brain with high number of viral copies (Chen et al., 2017).

Last but not least, a number of published studies found PCV-3 in apparently healthy animals (Franzo et al., 2018b; Ye et al., 2018; Zhai et al., 2017; Zou et al., 2018), which makes much more complicate the overall interpretation of this virus as potential causative agent of disease. Nevertheless, in some of them, the prevalence in diseased pigs (when studied together with healthy ones) was higher than in non-clinically affected pigs.

Table 1-4. Clinical signs reported in PCV-3 PCR-positive animals according to production phase and different clinical/pathological condition.

Disorders	Production phase	Clinical signs- Diseased animals	Control group- Healthy animals	Reference
Reproductive	Sows	• Increase in the sow mortality; decrease in the conception rates; mummified fetuses	NA	Palinski et al., 2017
		• Aborted fetuses, stillborn	NA	Faccini et al., 2017
		• Abortion, mummified fetuses; reproductive failure; decrease of neonatal rate	NA	Ku et al., 2017
Respiratory	Lactation	• Dyspnea	NA	Phan et al., 2017
	Weaning	• Anorexia, fever, ochrodermia, abdominal breathing	NA	Shen et al., 2017 Zhai et al., 2017
	Weaning	• Cough, softly panting, abdominal breathing	*	Phan et al., 2017
	Fattening	• Porcine respiratory disease complex (PRDC)	NA	Kedkovid et al., 2018

Table 1-4 (continuation). Clinical signs reported in PCV-3 PCR-positive animals according to production phase and different clinical/pathological condition.

Disorders	Production phase	Clinical signs- Diseased animals	Control group- Healthy animals	Reference
Cardiovascular	Weaning	• Anorexia, weight loss, swollen joints	NA	Phan et al., 2016
Gastrointestinal	Weaning	• Diarrheal	*	Zhai et al., 2017
Systemic	Weaning	• PCVD	*	Stadejek et al., 2017
Neurological	Lactation	• Neurological signs	NA	Phan et al., 2017
	Lactation	• Congenital tremors	NA	Chen et al., 2017
Others	Fattening	• Rectal prolapse	NA	Phan et al., 2017
	Sows	• PDNS	NA	Palinski et al.., 2017

NA: not available in the published study; *: PCV-3 positivity in lower frequency than diseased animal

Whilst the initially PCV-3 PCR positive samples were negative for three of the most important swine infectious agents i.e. PCV-2, PRRSV and PPV (Palinski et al., 2017; Phan et al., 2016; Zhai et al., 2017), subsequent studies revealed co-infection with other viruses. All pathogens found in co-infections with PCV-3 are summarized in Table 1-5.

Table 1-5. Pathogens present in PCV-3 PCR positive samples.

Pathogen	**Frequency of co-infection (percentage)**	**Reference**
PCV-2	38/200 (19%) 28/40 (70%) 35/222 (15.77%) 13/46 (28.26%) 1/8 (12.5%)	Sun et al., 2018 Zhao et al., 2018 Ku et al., 2017 Kim et al., 2017 Kedkovid et al., 2018b
PRRSV	1/8 (12.5%)	Kedkovid et al., 2018b
Torque teno sus virus (TTSuV1 and 2)	66/132 (50%)	Zheng et al., 2018
*Classical swine fever virus (*CSFV)	108/200 (54%)	Sun et al., 2018
*Porcine bocavirus (*PBoV)	NA	Chen et al., 2017
Porcine epidemic diarrhoea virus (PEDV)	NA	Chen et al., 2017
Atypical porcine pestivirus (APPV)	NA	Chen et al., 2017
*Porcine deltacoronavirus (*PDCoV)	NA	Chen et al., 2017
*Porcine kobuvirus (*PKV)	NA	Chen et al., 2017
Porcine pseudorabies virus (PRV)	NA	Chen et al., 2017
Porcine sapelovirus (PSV)	NA	Chen et al., 2017
Pasteurella multocida	NA	Kedkovid et al., 2018b
Haemophilus parasuis	NA	Phan et al., 2017
Streptococcus suis	NA	Phan et al., 2017
Mycoplasma hyorhinis	NA	Phan et al., 2017

NA: not available in the published study

1.5.4. Virus/antibody detection methods

As expected for any novel virus, detection and characterization of PCV-3 is to-date based on molecular techniques such as NGS, PCR, qPCR and Sanger sequencing. For PCR and qPCR purposes, a variety of primer pairs and probes have been designed (Chen et al., 2017; Franzo et al., 2018a; Palinski et al., 2017). In addition, duplex qPCR for the simultaneous detection of PCV-2 and PCV-3 has also been established (Li et al., 2018a).

With the aim to obtain partial or complete PCV-3 sequences, several primers pairs have also been designed; however, in a number of cases there is no possible to obtain complete sequences because of the limited quantity of DNA (Fux et al., 2018). In the first descriptions, metagenomic sequencing was performed while the subsequent sequences were obtained through traditional Sanger sequencing.

For the purpose to detect PCV-3 in histological sections of tissues, an *in situ* hybridization has been performed in few studies (Kedkovid et al., 2018b; Phan et al., 2016). However, the technique is not yet well developed, since it is still used in minimal number of laboratories worldwide and a thorough description of the positive cell types is still missing.

Also, the need of serological tests to measure antibody responses against PCV-3 is paramount. Only two reports have been published little information about indirect enzyme-linked immunosorbent (ELISA) tests using recombinant PCV-3 *cap* protein (Deng et al., 2018; Palinski et al., 2017). More recently, a PCV-3 specific monoclonal antibody has been produced, presumably working on formalin-fixed, paraffin-embedded tissues by means of immunohistochemistry (Li et al., 2018a).

Isolation of PCV-3 has been attempted in PK-15 (Faccini et al., 2017; Palinski et al., 2017) and swine testicle cells (ST) (Palinski et al., 2017). The cells were observed for cytopathic effects and monitored by qPCR for viral

growth. However, the Ct values did not increase at each cellular passage and no cytopathic effect was observed (Faccini et al., 2017; Palinski et al., 2017). Therefore, there is not any PCV-3 isolate so far available.

Definitely further studies are necessary to establish laboratory techniques such as viral isolation, serology and detection of virus elements in tissues in order to elucidate the pathogenesis of the PCV-3. Moreover, the potential association of PCV-3 with any clinical condition, if any, is still to be demonstrated.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

Porcine circoviruses are important pathogens in the pig population. PCV-1 is considered a non-pathogenic virus (Tischer et al., 1986), but PCV-2 is one of the most important infectious agents causing clinical and subclinical disorders in swine (Segalés et al., 2013). In 2016, a novel PCV, named PCV-3, was detected by NGS and found in samples from healthy and diseased animals. Moreover, PCV-3 is spread worldwide and reports showed that the virus is not new, but just a recently discovered virus. Whether PCV-3 is pathogenic or simply ubiquitous in nature without causing major problems in swine is still not known. Moreover, it is difficult to predict the ability of the virus to cause disease since the viral isolation in tissue samples has not been successful so far (Palinski et al., 2017), and no disease reproduction has been attempted.

Taking into account the impact of PCV-2 over the swine production in the last 20 years worldwide, the advent of a third PCV in 2016, found initially in cases of disease, drove attention the to the veterinary and scientific community. Therefore, investigations on the new virus were needed. Particularly, its truly association with disease, development of diagnostic methods, epidemiology and pathogenesis are fields of major interest for an emerging virus.

At the beginning of the present PhD Thesis, very limited information about the virus was available (only two articles were published at that time, Palinski et al., 2017 and Phan et al., 2016). Therefore, the present Thesis aimed to gain insights into the molecular epidemiology of PCV-3 in samples from domestic pigs and wild boar from Spain. The specific objectives were:

- To evaluate retrospectively the presence of PCV-3 in serum samples collected between 1996 and 2017 (*Chapter3)*

- To study the dynamics of PCV-3 infection in a set of clinically healthy domestic pigs longitudinally sampled from conventional farms *(Chapter 4)*

- To investigate different aspects of PCV-3 infection in wild boar, including a retrospective study, and assessing the dynamics of infection and tissue distribution (*Chapter 5).*

CHAPTER 3

STUDY I

Retrospective detection of *Porcine circovirus 3* (PCV-3) in pig serum samples from Spain

Published at Transboundary and Emerging Diseases. 2018.
Doi: 10.1111/tbed.12876.

3.1. INTRODUCTION

PCV-3 is the third circovirus type found in swine. PCV-1 was the first described member of the *Circoviridae* family around 40 years ago (Tischer et al., 1982, 1974), which has been historically considered non-pathogenic for swine (Allan et al., 1995). By mid-late 1990s, PCV-2 was discovered in association with the so-called postweaning multisystemic wasting syndrome (PMWS), nowadays known as PCV-2-systemic disease, PCV2-SD (Segalés, 2012). PCV2-SD was considered a devastating disease during more than a decade (Segalés et al., 2013), although it is nowadays under control as a result of vaccination (Segalés, 2015). In spite of being discovered by late 1990s, PCV-2 was circulating in the swine population long time before; retrospective studies have shown that PCV-2 was present in pigs as early as 1962 and PCV2-SD already existed by mid-1980s (Jacobsen et al., 2009). Taking into account the natural history of previously known porcine circoviruses, it is very likely that PCV-3 has also been present within the swine population before its initial detection in 2016.

Therefore, the aim of the present work was to perform a retrospective study to detect evidence of PCV-3 infection in Spanish serum samples of pigs collected between 1996 and 2017. Moreover, the genetic characterization of PCV-3 during the 22-year study period based on partial sequences of the viral genome is provided.

3.2. MATERIAL AND METHODS

3.2.1. Retrospective swine sera

Serum samples stored at -20°C from 654 pigs submitted for diagnostic purposes at the Veterinary Pathology Diagnostic Service of the *Universitat Autònoma de Barcelona* (Barcelona, Spain) between 1996 and 2017 were used for this study. From 1996 to 2005, 17 to 20 sera were randomly selected from

the serum bank, except for year 1996, where only 13 serum samples were available; between 2006 and 2017, 26-74 sera were chosen. Sera corresponded to animals from different, non-related diagnostic studies performed across years and information about the overall production phase and clinical/pathological status of tested pigs is summarized in Tables 3-1 and 3-2, respectively.

Table 3-1. Number of PCV-3 in serum samples from 1996 to 2017 tested by conventional PCR.

Year	Number of examined cases (n)	PCV-3 PCR positive cases (n)	Percentage (%)
1996	13	1	7.69
1997	20	2	10.00
1998	20	1	5.00
1999	19	1	5.26
2000	19	1	5.26
2001	20	2	10.00
2002	20	3	15.00
2003	20	1	5.00
2004	20	1	5.00
2005	17	0	0.00
2006	39	2	15.38
2007	39	6	15.38
2008	40	7	17.50
2009	26	0	0.00
2010	40	6	15.00
2011	39	3	7.69
2012	37	9	24.32
2013	22	3	13.64
2014	42	7	16.67
2015	41	8	19.51
2016	27	1	3.70
2017	74	10	13.51
Total	654	75	11.47

Table 3-2. Frequencies of PCV-3 positive and negative samples grouped according to different clinical/pathological presentations and production phase.

Clinical/pathological disorders																
Prod. phase	PCV-3 PCR Results	Tot *	Gastro.		Neuro.		Respirat.		Repro.		Systemic		Others		Asympt.	
		N	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Foetuses	Positive	3	0	0.0	0	0	0	0.0	3	100	0	0.0	0	0.0	0	0.0
	Negative	3	0	0.0	0	0.0	0	0.0	3	100	0	0.0	0	0.0	0	0.0
Lactation	Positive	10	1	10.0	2	20.0	5	50.0	0	0	1	10.0	1	10.0	0	0.0
	Negative	113	29	25.7	22	19.5	6	5.3	0	0	19	16.8	37	32.7	0	0.0
Nursery	Positive	36	10	27.8	2	5.6	9	25.0	0	0	10	27.8	2	5.6	3	8.3
	Negative	262	49	18.7	16	6.1	67	25.6	0	0	74	28.3	30	11.5	26	9.9
Fattening	Positive	21	1	4.8	2	9.5	3	14.3	0	0	10	47.6	1	4.7	4	19.0
	Negative	172	13	7.6	7	4.1	35	20.3	0	0	67	38.9	10	5.8	40	23.3
Sows	Positive	0	0	0.0	0	0.0	0	0.0	0	0	0	0.0	0	0.0	0	0.0
	Negative	5	0	0.0	0	0	1	20.0	0	0	1	20.0	3	60.0	0	0.00
Total*	Positive	70	12	17.1	6	8.6	17	24.3	3	4.3	21	30.0	4	5.7	7	10.0
	Negative	555	91	16.4	45	8.1	109	19.6	3	0.5	161	29.0	80	14.4	66	11.9
	Total	625	103	16.5	51	8.2	126	20.2	6	0.9	182	29.1	84	13.4	73	11.68
*** No information was available for 5 out 75 PCV-3 PCR positive animals and 24 out of 579 for PCV-3 negative animals**																

Prod.: Production; Tot: Total; Gastro: Gastrointestinal; Neuro.: Neurological; Respirat.: Respiratory; Repro.:Reproductive; Asympt.: Asymptomatic

3.2.2. DNA extraction and polymerase chain reaction (PCR) to detect and sequence PCV-3

DNA from 200 µL of serum samples was extracted using MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol. A plasmid containing the full-length PCV-3 sequence (Franzo et al., 2018a) and double distilled water were used as positive and negative controls, respectively. To demonstrate the presence of PCV-3 DNA in studied samples, a conventional PCR assay was performed; according a previous protocol described (Franzo et al., 2018b), three µl of extracted DNA was added to a PCR mix and amplified using the same thermal protocol. The reaction was carried out in a final volume of 50 µl mixture containing 10µl of 5x PCR Buffer, 2 µl of 10 µM dNTPs, 1 µl of 10 pmol forward primer located in the genomic positions 233-255, 5'-AAAGCCCGAAACACAGGTGGTGT-3', 1 µl of 10 pmol of a reverse primer 5'-TTTTCCCGCATCCTGGAGGACCAAT- 3' situated between nucleotide positions 718-742, 1 Units of DNA polymerase Platinum™ SuperFi™ (Invitrogen™) and 32.5 µl of water. For the partial genome sequence amplification, two specific primer pairs able to detect two overlapping amplicons were used, a forward 5'-CACCGTGTGAGTGGATATAC-3' and reverse primer 5'-CAAACCCACCCTTAACAG-3' (located in the genomic positions 74-93 and 909-927, respectively); forward primer 5'-GTCGTCTTGGAGCCAAGTG-3' and reverse 5'-CGACCAAATCCGGGTAAGC-3' (situated between positions 1612-1627 and 415-433, respectively) (Palinski et al., 2017). The extracted DNA was added to a same quantity of mix as reported above applying as well the same PCR conditions. The PCR products were run on 1.8% TAE agarose gel and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)

according to the manufacturer's protocol. The quality and quantity of genomic DNA was analysed with BioDrop DUO (BioDrop Ltd).

Twelve samples from different years were then Sanger sequenced for verification at the Genomic and Bioinformatics Service of the *Universitat Autònoma de Barcelona* (Barcelona, Spain), which was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit, following the manufacturer's protocol. The sequencing reactions were analyzed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®)**.**

3.2.3. Sequence alignment and phylogenetic analysis

Sequence analysis was performed with Finch TV program 1.4.1 (http://www.geospiza.com), the consensus was obtained utilizing ChromasPro (CromasPro Version 1.5) and sequences were aligned with 51 PCV-3 complete genomes available at the GenBank (retrieved on November 2017) using MAFFT (Kumar et al., 2016). A phylogenetic tree was conducted using the Maximum-Likelihood (ML) method implemented in PhyML (Guindon et al., 2010); the robustness of the ML tree was evaluated by bootstrap analysis with 1,000 bootstrap replicates. The raw genetic distance among strains was calculating using MEGA7 software (Kumar et al., 2016). Sequences obtained are available at GenBank (references MG807066 to MG807089).

3.2.4. Association between presence of PCV-3 and production phase and clinical/pathological conditions

All studied animals were classified according to their respective production phases (lactation, from 1 to 3 weeks of age; nursery, from 4 to 9 weeks of age; fatteners, >10 weeks of age; sows and foetuses were also included as a farrowing category). Animals were still classified according to

their different clinical/pathological presentations (gastrointestinal, systemic, neurological, reproductive, respiratory and other conditions such as cutaneous, musculoskeletal or inconclusive clinical signs). A number of asymptomatic animals were included as well. Table 3-2 summarizes the number of pigs in each production phase as well as their clinical/pathological presentation. The correlation between PCV-3 and PCV-2 or PRRSV was also described.

3.2.5. Statistical analyses

The statistical analyses were performed using XLSTAT 365 Microsoft Excel 2016 Statistics Software. To test for significant differences between production phases, the corresponding clinical/pathological status and the PCV-3 PCR positivity frequencies across the 22 years tested in this study, a Fisher's exact test was performed. P values <0.05 were considered to be statistically significant.

3.3. RESULTS

3.3.1. PCV-3 detection by PCR

PCV-3 PCR positivity was found in 75 out of 654 (11.46%) serum samples. The first PCV-3 PCR positive sample dated back to 1996, and the viral genome was subsequently detected in sera from all tested years except 2005 and 2009. The frequency in the positive years ranged from 3.70% (1 out of 27) in 2016 to 24.32% (9 out of 37) in 2012 (Table 3-1). No statistical differences in the frequency of PCV-3 positive animals were observed across the years tested ($p>0.05$).

3.3.2. Sequence alignment and phylogenetic analysis

Partial sequences were obtained from twelve PCV-3 PCR positive samples from different years and comprised part of the rep (848 to 949 nucleotide positions) and cap (338 to 387 nucleotide positions) genes. The phylogenetic analysis (Figure 3-1) showed more than 98% of identity among the Spanish strains and also between the Spanish strains and other already published PCV-3 sequences. The oldest PCV-3 partial sequences obtained were from 1997 (GenBank references MG807066 for Rep gene and MG807078 for Cap gene), which shared a 98% nucleotide identity with the first PCV-3 sequence described in the USA in 2016 (KX898030.1).

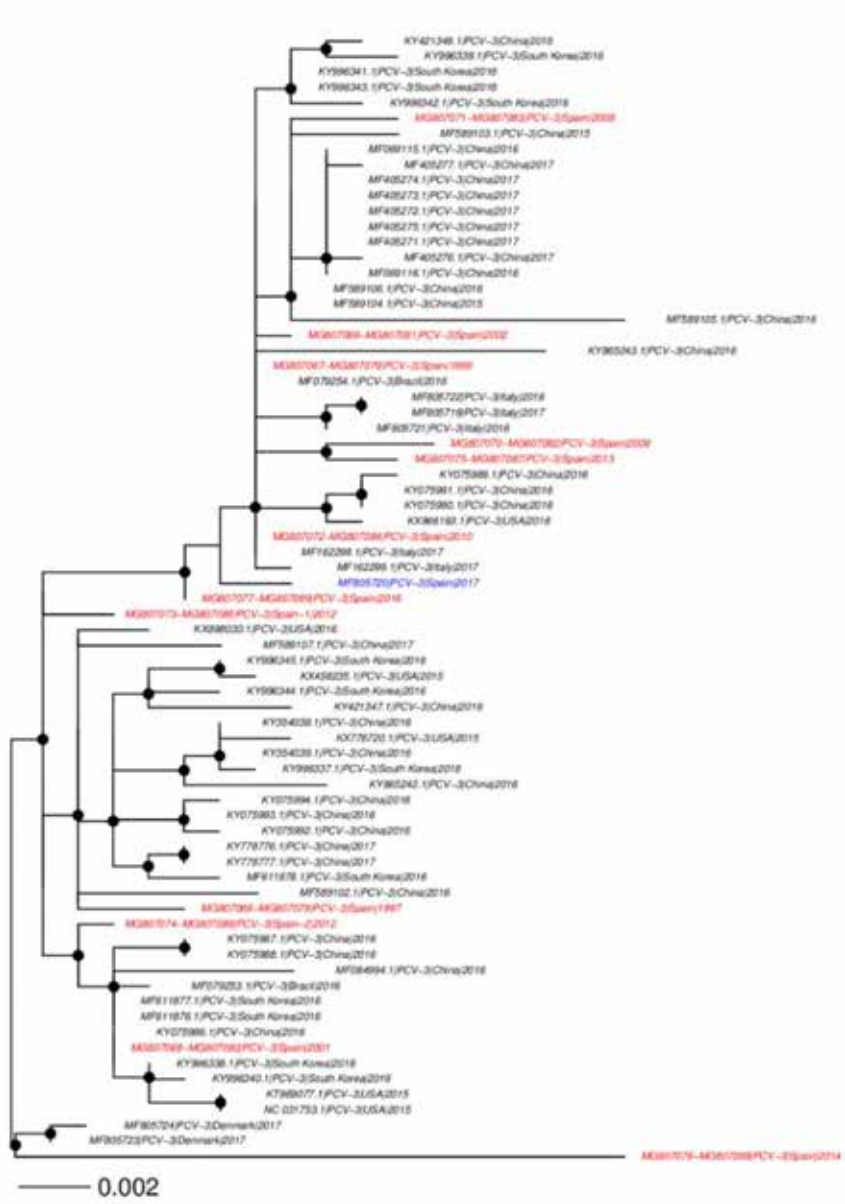

Figure 3-1. Phylogenetic tree of the partial genomes of PCV-3 Spanish strains and PCV-3 freely available sequences at GenBank. Phylogenetic tree was constructed using the maximum-likelihood algorithm of PhyML software with 1,000 bootstrap replicates. The colour blue indicates a previous PCV-3 Spanish sequence reported by Franzo *et al.* (2018a) the red lines indicates the PCV-3 Spanish samples obtained in this work. Nodes demonstrating a branch support higher than 70% have been marked with a full circle.

3.3.3. Association between presence of PCV-3, production phase and clinical/pathological conditions

The detection of PCV-3 genome split by production phase and clinical/pathological conditions is summarised in Table 3-2. No information about production phase and clinical/pathological condition was available for 5 out of 75 PCV-3 PCR positive pigs and 24 out of 579 negative ones.

In total, 37 PCV-3 PCR positive animals were tested for PCV-2 and 45 for PRRS. The frequency ranged from 24.32% (9 out of 37) and 15.55% (7 out of 45) for PCV-2 and PRRSV, respectively. Overall, 36 out of 298 nursery pigs (12.08%), 21 out of 193 (10.88%) fatteners and 10 out of 123 (8.13%) lactating pigs were PCV-3 PCR positive, no statistically significant differences were observed among production phases ($p>0.05$). None of the five sows tested were PCV-3 PCR positive; in contrast, 3 out of 6 (50%) foetuses were positive.

According to the different clinical/pathological conditions, 17 out of 126 (13.49%) pigs with respiratory disorders were PCV-3 PCR positive as well as 21 out of 182 pigs (11.53%) with systemic problems and 12 out of 103 pigs (11.65%) with gastrointestinal disorders. Likewise, the positivity rate was identified in 6 out of 51 pigs (11.76%) with neurological and 3 out 6 (50%) aborted foetuses. Also, 4 out of 84 (4.76%) pigs included in the group of other conditions and 7 out of 73 (9.58%) asymptomatic animals were PCV-3 PCR positive. No statistically significant differences were observed among clinical/pathological conditions ($P>0.05$).

3.4. DISCUSSION

PCV-3 was first reported in 2016. Since then, many investigations have shown the presence of its genome in pig samples from different production phases, affected by a variety of pathological disorders, and also distributed in

different countries worldwide. A recent study indicated the presence of PCV-3 genome as early as 2002 in tissue and faecal samples in Northern Ireland (Collins et al., 2017), suggesting that PCV-3 is not a new virus and has probably been circulating for a relatively extended period in pig populations around the world. The present retrospective investigation further confirms such a hypothesis; PCV-3 has been circulating in the Spanish pig population at least since 1996, the earliest year of detection of PCV-3 worldwide to date.

Compiled data along 22 years offered a mean PCV-3 PCR positive rate of 11.47%. These data agree with the detection frequency found in Spanish serum samples (14 out of 94; 15%) from a previous study, whilst they are slightly lower than those obtained in Italy (18.18%), Poland (25%), Denmark (30%), South Korea (44.20%) and China (59.46%) (Stadejek et al., 2017; Zheng et al., 2017; Franzo et al., 2018a). The study design of the mentioned works were different in relation to both, the studied age categories and PCR methodologies used, implying that a direct comparison of positivity frequency is not feasible. Therefore, the prevalence of the virus in the different geographical regions cannot be accurately stated at present.

The phylogenetic analysis of obtained sequences in the present retrospective study demonstrates a close distance between them and with the PCV-3 genomes available at the GenBank. Even if the genetic variability was globally low, the Spanish strains were intermingled amongst all currently available PCV-3 sequences in the phylogenetic tree. This result would suggest that PCV-3 has not shown a differentiated independent molecular evolution in the particular areas of the world where it has been detected so far. Moreover, the low genetic variability found in PCV-3 would point to a recent emergence (and consequently, a relatively short time to evolve) or, alternatively, to a slow evolution rate as has been found for PCV-1 (Cortey & Segalés, 2011). Further

phylogenetic and evolutionary studies with higher numbers of PCV-3 sequences are needed to ascertain those hypotheses.

Some studies identified the presence of PCV-3 in several tissue samples or sera from pigs with different clinical presentations (Palinski et al., 2017; Tochetto et al., 2017; Zhai et al., 2017), as well as in healthy animals (Zheng et al., 2017; Franzo *et al.,* 2018). The data compiled in this study suggest a homogeneous PCV-3 frequency in the different production phases and clinical/pathological conditions, since no statistically significant differences were found among tested groups. Previous studies reported co-infections of PCV-3 with PCV-2 (Fu et al., 2017; Ku et al., 2017) and PRRSV (Fu et al., 2017), two of the most important pathogens affecting pigs worldwide. In the studied animals, PCV-2 and PRRSV were investigated for diagnostic purposes attending to the demand of the submitting veterinarian. In total, 9 out of 37 (24.32%) and 7 out of 45 (15.55%) of the PCV-3 PCR positive cases were also positive for PCV-2 and PRRSV, respectively. It is very likely that those percentages of co-infections reflect the relatively widespread nature of all these three viruses in Spain, rather than a potential synergism or association.

Noteworthy, at least for PCV-2, the link between clinical disease and the virus is associated with the viral load; the higher the viral load, the higher the probability of the animal being sick (Grau-Roma et al., 2009). Specifically, some reports have shown significant differences in PCV-2 viral load in serum samples, as well in different tissues, between healthy and clinically sick PCV-2-SD pigs; loads above 10^7 were detected in diseased animals (Brunborg et al., 2007; Olvera et al., 2004; Segalés and Domingo, 2002). Recently, Zhai et al. (2017) found lower qPCR Ct values in samples from animals with respiratory signs and diarrhoea compared to those of asymptomatic animals, suggesting that PCV-3 may be potentially associated with these clinical conditions. A recently described qPCR for PCV-3 detection (Franzo et al., 2018a) was

attempted in the present study with a limited number of positive samples as an exploratory approach (data not shown). Results showed Ct values ranging from 24.23 to 39.04 (equivalent to $10^7$and 10^2 PCV-3 genome copies/mL of serum), but no apparent correlation with the clinical/pathological conditions was found.

Definitively, more studies trying to confirm or rule out a potential relationship of PCV-3 or certain PCV-3 loads with disease in swine are needed.

In conclusion, the current study confirms PCV-3 circulation in the Spanish pig population with a low/moderate frequency, at least since 1996. The high identity among PCV-3 partial genome sequences indicates that this virus has remained relatively stable across the years. Preliminarily, PCV-3 infection did not appear to be linked to any specific pathological condition nor particular pig age-group or production phases.

CHAPTER 4

STUDY II

Infection dynamics of *Porcine circovirus 3* in longitudinally sampled pigs from four Spanish farms

Submitted for publication

4.1. INTRODUCTION

Since the first description in North America (Palinski et al., 2017; Phan et al., 2016), many reports have identified PCV-3 in Europe (Faccini et al., 2017; Franzo et al., 2018b; Stadejek et al., 2017), Asia (Hayashi et al., 2018; Ku et al., 2017; Kwon et al., 2017; Shen et al., 2017) and South America (Saraiva et al., 2018; Tochetto et al., 2017), suggesting a worldwide circulation. Moreover, retrospective studies have shown PCV-3 circulation at least since the 1990s (Sun et al., 2018; Ye et al., 2018; Study I of this Thesis) and, according to phylogenetic analyses, the common ancestor was dated around 50 years ago (Fu et al., 2017; Saraiva et al., 2018).

The first metagenomics analyses revealed PCV-3 genome in sows with porcine dermatitis and nephropathy disease (PDNS) and chronic reproductive failure (Palinski et al., 2017). Subsequently, PCV-3 was found in tissue homogenates in pigs with a causally unexplained myocarditis (Phan et al., 2016). Thereafter, reports identified PCV-3 genome in nursery and fattening pigs with different clinical/pathological presentations as respiratory disorders (Sun et al., 2018; Zhai et al., 2017) and in neonatal piglets with congenital tremors (Chen et al., 2017). In addition, the genome was detected in apparently healthy sows and fattening pigs as well as in stillborns (Franzo et al., 2018c; Zhai et al., 2017; Zheng et al., 2017). To date, it is not demonstrated whether PCV-3 infection is linked to a particular pathological condition or any specific age (Kwon et al., 2017; Stadejeck et al., 2017).

Based on available literature, it looks evident that PCV-3 is present in almost all pig ages (from fetuses to adults). However, a comprehensive study of the infection dynamics of this virus in a pig healthy population has not been described so far. Therefore, the aim of the present study was to longitudinally assess the dynamics of PCV-3 infection in a set of pigs from four clinically healthy conventional farms from Spain.

4.2. MATERIAL AND METHODS

4.2.1. Study design

Serum samples corresponding to 152 pigs from four selected clinically healthy conventional farms from Spain were chosen for this study (Table 4-1). Samples were collected during years 2012 and 2016 for different study purposes (Feng et al., 2016 and unplublished data; Fraile et al., 2012; Oliver-Ferrando et al., 2016). In the first farm (Farm A), 34 piglets were sampled longitudinally at 2, 8, 13, 18 and 24 weeks of age. In farm B, 44 piglets were sampled at 2, 7, 12, 18, 22 and 25 weeks of age. From farm C, 28 animals were followed up at 2, 6, 10, 14, 18 and 25 weeks. Finally, 46 piglets were longitudinally sampled at 4, 8, 12, 16, 21 and 25 weeks of age from farm D. The weeks were grouped according to the production phase (lactation, from 1 to 4 weeks of age; nursery, from 5 to 9 weeks of age; and growing/fattening; >10 weeks of age) (Figure 4-1).

Table 4-1. Production system, farm size and vaccination programs applied in piglets and sows in the farms under study.

Farm ID	Production system	Herd size	Sow vaccination program*	Piglet vaccination program*
Farm A	Two-site, AI-AO	1,800 sows	ADV, PPV, Ery, EC, CP, PRRSV	PCV-2, Mhyo
Farm B	Multi-site, AI-AO	3,300 sows	ADV, PPV, Ery, EC, CP	PCV-2, Mhyo
Farm C	Two-site, AI-AO	800 sows	ADV, PPV, Ery, EC, CP, PRRSV, SIV,	Mhyo
Farm D	Two-site, AI-AO	1,500 sows	ADV, PPV, Ery, EC, CP, PRRSV	Mhyo

AI-AO: all in-all out management practices.*ADV: *Aujeszky's disease virus*; PRRSV: *Porcine reproductive and respiratory syndrome virus*; PPV: *Porcine parvovirus*; PCV-2: *Porcine circovirus 2*; SIV: *Swine influenza virus*; Ery: *Erysipelothrix rhusiopathiae*; Mhyo: *Mycoplasma hyopneumoniae*; EC: *Escherichia coli*; CP: *Clostridium perfringens*

4.2.2. DNA extraction and specific polymerase chain reaction (PCR) for PCV-3 detection and sequencing

DNA was extracted from 200 µL of serum using MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol. Double distilled water and a plasmid containing the full-length PCV-3 genome included into a PCV-3 negative serum (Franzo et al., 2018a) were used as negative and positive controls, respectively.

To detect the presence of PCV-3 DNA in tested samples, a conventional PCR assay was performed based on a previous protocol described

by Franzo et al. (2018a), with slight modifications. Three µL of extracted DNA were added to a PCR mix and amplified using the described thermal protocol. The reaction was carried out in a final volume of 50 µL mixture containing 1x PCR Buffer, 10 pmol of dNTPs, 10 pmol of forward primer located in genomic positions 233-255 (5'- AAAGCCCGAAACACAGGTGGTGT-3'), 10 pmol of reverse primer placed between nucleotide positions 742 and 718 (5'-TTTTCCCGACATCCTGGAGGACCAAT- 3'), one EU of DNA polymerase Platinum™ SuperFi™ (Invitrogen™) and double distilled water.

For sequencing purposes, the extracted DNA from PCV-3 PCR positive samples was amplified as described above, using as forward primer 5'-CACCGTGTGAGTGGATATAC- 3' and reverse primer 5'-CACCCCAACGCAATAATTGTA- 3' (located in the genomic positions 74-94 and 1,144-1,123, respectively) under the thermal conditions described by Fux et al. (2018). In order to increase the amount of amplicon to be sequenced the PCR products were re-amplified with the same protocol. All PCR products were electrophoretically separated on 1.2% TAE agarose gel. The PCV-3 PCR-positive samples were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) according to the manufacturer's protocols and the quality and quantity of genomic DNA was analysed with BioDrop DUO (BioDrop Ltd).

4.2.3. Sequence analyses

PCV-3 positive samples were selected and submitted to Sanger-sequencing, which was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit, following the manufacturer's protocol at the Genomic and Bioinformatics Service of the *Universitat Autònoma de Barcelona* (Barcelona, Spain). The sequencing reactions were analysed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®)**.**

Sequences and chromatograms were manually explored to trim bad-quality bases with BioEdit 7.2 (Hall, 1999). The assembly of the consensus sequences extracted from different fragments was attempted using DNASTAR Lasergene software (Burland, 1999). The partial genomes obtained were aligned using Clustal Omega (Thompson et al., 1997) with 74 complete genome sequences available at the GenBank (Table 4-2) and trimmed accordingly for comparison purposes. A phylogenetic tree was constructed with the Maximum-Likelihood (ML) method based on the best predicted-substitution model (lowest BIC score) by means of the Tamura-Nei plus Gamma substitution model (Tamura and Nei, 1993) using MEGA software version 7 (Kumar et al., 2016). The robustness of the clade was evaluated with 1,000 bootstrap replicates. The obtained sequences were submitted at the GenBank (references MH780665- MH780672).

4.2.4. Statistical analyses

Statistical analyses were performed using XLSTAT 365 Microsoft Excel 2016. To test for significant differences between weeks of age in each tested farm, the Fisher's exact test was performed. The significance level was set as 0.05.

Table 4-2. List of sequences included in the analysis according to the country and collection year.

Genbank acession no	Collection country	Collection year
MH579736-MH579738	Spain	2005-2007
MH579739-MH579743	Spain	2010-2014
MG014365	Germany	2015
MG014375	Germany	2015
MG014364	Germany	2015
MG014373	Germany	2015
MG014370	Germany	2015
MG014369	Germany	2015
MG014376	Germany	2015
MG014362	Germany	2015
MG014372	Germany	2015
MG014368	Germany	2015
MG250181	Germany	2015
MG014367	Germany	2015
MG014366	Germany	2015
MG014363	Germany	2015
MG014371	Germany	2015
MG014374	Germany	2015
KX458235	USA	2015
KX778720	USA	2015
KT869077	USA	2015
MH579744	Spain	2015
MF079254	Brazil	2016
MF079253	Brazil	2016
KY075990	China	2016
KY075991	China	2016
KY075989	China	2016
KY865243	China	2016
MG564175	China	2016
KY418606	China	2016
MG250179	China	2016
MG250176	China	2016
MG250177	China	2016
KY075992	China	2016

Table 4-2 (continuation). GenBank accession number of sequences included in the analysis according to the country and collection year.

Genbank acession no	Collection country	Collection year
KY075993	China	2016
KY075994	China	2016
KY865242	China	2016
KY996340	China	2016
KY075986	China	2016
KY075987	China	2016
KY075988	China	2016
MF318448	China	2016
MF318449	China	2016
MF318450	China	2016
KY996341	South Korea	2016
KY996343	South Korea	2016
KY996339	South Korea	2016
KY996342	South Korea	2016
KY996344	South Korea	2016
KY996345	South Korea	2016
KY996337	South Korea	2016
KY996338	South Korea	2016
KX966193	USA	2016
KX898030	USA	2016
MH579745	Spain	2016
MG250182	China	2017
MG250180	China	2017
MG250187	China	2017
MG250183	China	2017
MG250184	China	2017
MG250185	China	2017
MG250186	China	2017
KY778776	China	2017
KY778777	China	2017
MG250178	China	2017
MF805720	Spain	2017
MH579746	Spain	2017
MH579747	Spain	2018

4.3. RESULTS

4.3.1. PCV-3 detection by PCR

PCV-3 genome was detected in all tested farms and sampling points during the study period.

Overall, PCV-3 PCR positivity was found in 28 out of 34 (82.35%), 32 out of 44 (72.72%), 22 out of 28 (78.57%) and 34 out of 46 (71.74%) pigs in farms A, B, C and D, respectively. Results of the PCV-3 prevalence obtained by PCR in each age-group are summarised in Figure 4-1. Individual PCR results for each pig from each farm are displayed in Table 3-1.

Globally, the PCV-3 positive percentage was fairly uniform within each tested farm (Figure 4-1). In farm A, PCV-3 DNA detection prevalence ranged from 23.53% (8 out of 34 pigs) at the second sampling to 32.35% (11 out of 34 animals) at the last one. In farm B, PCV-3 genome presence varied from 9.09% (4 out of 44, first sampling) to 36.37% (15 out of 44, fifth sampling). Such frequency ranged from 10.71% (3 out of 28, fifth sampling) to 34.71% (10 out of 28, fourth sampling) in farm C, and from 6.52% (3 out of 46, third sampling) to 34.78% (16 out of 46, second sampling) in farm D. No statistically significant differences were found across the tested weeks of age ($p>0.05$) in farms A and C; however, differences in PCV-3 prevalence were detected among tested ages in farms B and D (Figure 4-1).

Detection results of individual PCV-3 PCR for each farm are depicted in Tables 4-3a to 4-3d. In most of the cases, the detection of PCV-3 was either intermittent or found once in life. In farm A, 3 out of 28 (10.7%) animals showed infection intermittently and 10 animals (35.71%) had a continuous PCR-positive result during a period ranging from 5 to 22 weeks; only one pig was positive at all sampling times. In farm B, intermittent detection of PCV-3 was observed in 10 out of 44 animals (22.7%); 8 more pigs (18.18%) showed

continuous PCR positivity during a period of 4 to 23 weeks; again, one of them was PCV-3 PCR positive at all sampling points. In farm C, 8 out of 28 (28.6%) animals had PCV-3 DNA in serum intermittently and only two more animals (7.14%) were positive during two consecutive samplings. Finally, in farm D, most pigs were PCV-3 PCR positive once during the study period (26 out of 46; 56.52%), 5 out of 46 (10.87%) had an intermittent detection of PCV-3 during a period from 5 to 17 weeks, and, finally, 3 more had continuous PCR PCV-3 detection ranging from 4 to 9 weeks. The numbers of animals PCV-3 PCR positive in more than one sampling are depicted in Table 4-4.

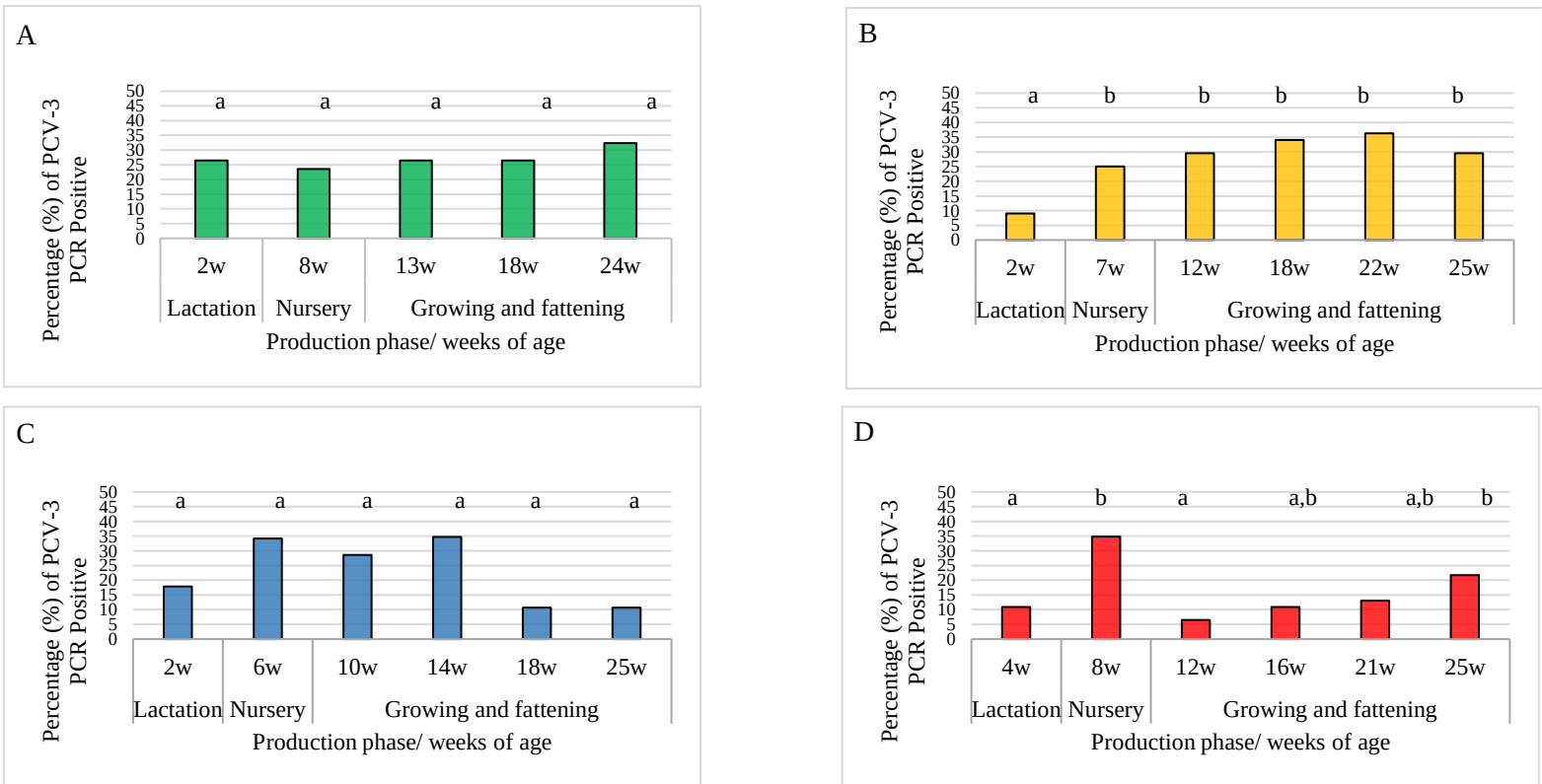

Figure 4-1. Percentage of PCV-3 frequency on tested farms distributed according to the analysed weeks of age and production periods for farms A (A), B (B), C (C) and D (D).

Table 4-3a. PCV-3 PCR positive (indicated as 1) and negative (indicated as 0) animals from each sampling in Farm A.

Animal	W1	W2	W3	W4	W5	W6
1	1	0	0	0	0	*
2	1	0	0	0	0	*
3	0	0	0	1	0	*
4	0	0	0	1	0	*
5	0	0	0	0	0	*
6	1	1	1	0	0	*
7	0	0	0	0	0	*
8	1	0	1	0	0	*
9	1	0	0	0	0	*
10	1	0	1	0	0	*
11	1	1	0	0	0	*
12	0	0	0	0	1	*
13	0	0	0	0	0	*
14	0	0	0	0	1	*
15	0	0	0	0	0	*
16	0	0	0	0	0	*
17	*	0	0	1	1	*
18	0	1	0	0	0	*
19	0	0	0	1	1	*
20	0	0	0	0	0	*
21	0	0	0	1	0	*
22	1	0	0	0	0	*
23	1	1	1	1	1	*
24	0	0	1	1	1	*
25	0	0	1	1	0	*
26	0	0	0	0	1	*
27	0	0	0	1	1	*
28	0	0	0	0	1	*
29	0	1	1	0	0	*
30	0	1	1	0	0	*
31	0	0	1	0	1	*
32	0	0	0	0	1	*
33	0	1	0	0	0	*
34	0	1	0	0	0	*

Table 4-3b. PCV-3 PCR positive (indicated as 1) and negative (indicated as 0) animals from each sampling in Farm B.

Animal	W1	W2	W3	W4	W5	W6
1	*	1	0	1	1	0
2	*	0	1	0	1	0
3	*	0	0	1	0	0
4	*	0	0	0	1	0
5	*	0	0	0	1	0
6	*	0	0	0	1	0
7	*	0	1	0	0	1
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	1
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	1	0	0	0
14	0	0	0	1	0	0
15	0	1	0	1	1	1
16	0	0	0	0	0	0
17	0	0	0	0	0	1
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	1	1	1	1	0	1
21	0	1	0	1	0	0
22	1	1	1	1	1	0
23	1	1	1	1	1	1
24	0	0	1	0	0	1
25	0	0	0	0	0	0
26	0	1	0	0	0	1
27	0	0	0	0	0	0
28	0	1	1	1	1	1
29	0	0	0	0	1	0
30	0	0	1	1	1	0
31	0	1	1	1	1	0
32	0	0	0	1	0	1
33	0	1	1	1	1	1
34	1	0	0	0	0	0

Table 4-3b (continuation). PCV-3 PCR positive (indicated as 1) and negative (indicated as 0) animals from each sampling in Farm B.

Animal	W1	W2	W3	W4	W5	W6
35	0	1	0	0	0	0
36	0	1	1	0	1	0
37	0	0	0	1	0	0
38	0	0	0	0	0	1
39	0	0	0	0	0	0
40	0	0	0	1	1	0
41	0	0	0	0	0	0
42	0	0	0	0	0	0
43	0	0	0	0	1	0
44	0	0	1	1	1	1

Table 4-3c. PCV-3 PCR positive (indicated as 1) and negative (indicated as 0) animals from each sampling in Farm C.

Animal	W1	W2	W3	W4	W5	W6
1	0	0	0	0	0	0
2	1	1	0	1	0	0
3	0	0	0	1	0	1
4	0	0	1	0	0	0
5	0	0	1	0	0	0
6	0	0	0	0	0	0
7	0	0	1	1	0	0
8	0	0	0	0	0	0
9	1	0	1	1	0	0
10	0	1	0	1	0	0
11	0	0	0	1	0	0
12	1	0	1	0	1	1
13	0	0	1	0	0	0
14	0	0	0	0	0	0
15	0	1	1	0	0	1
16	0	1	0	0	0	0
17	1	0	0	0	0	0
18	0	0	0	1	0	0
19	0	0	0	0	0	0
20	1	0	0	0	0	0
21	0	0	1	1	0	0
22	0	0	0	0	0	0
23	0	1	0	0	0	0
24	0	0	0	0	1	0
25	0	1	0	1	1	0
26	0	1	0	1	0	0
27	0	1	0	0	0	0
28	0	1	0	0	0	0

Table 4-3d. PCV-3 PCR positive (indicated as 1) and negative (indicated as 0) animals from each sampling in Farm D.

Animal	W1	W2	W3	W4	W5	W6
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	1
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	1	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	1	0	0
10	0	0	0	1	0	0
11	0	1	0	0	0	0
12	0	1	0	0	0	0
13	0	1	0	0	0	0
14	0	0	1	0	0	0
15	1	1	1	0	0	0
16	0	1	0	0	0	0
17	0	0	0	0	0	0
18	0	1	0	0	0	0
19	1	0	0	1	1	0
20	0	1	0	0	0	0
21	0	0	0	0	0	0
22	0	1	0	0	0	0
23	0	0	0	0	0	1
24	0	0	0	0	0	0
25	0	0	0	0	1	0
26	1	0	0	0	1	0
27	0	1	0	0	0	1
28	0	0	0	1	1	1
29	0	0	0	0	1	1
30	0	0	0	0	0	1
31	0	1	0	0	0	0
32	0	1	0	0	0	1
33	0	1	0	0	0	0
34	0	1	0	0	0	0

Table 4-3d (continuation). PCV-3 PCR positive (indicated as 1) and negative (indicated as 0) animals from each sampling in Farm D.

Animal	W1	W2	W3	W4	W5	W6
35	0	0	0	0	0	1
36	0	0	0	0	0	1
37	0	0	0	0	0	0
38	0	0	0	1	0	0
39	0	1	0	0	0	0
40	0	0	0	0	0	0
41	0	0	1	0	0	1
42	1	0	0	0	0	0
43	1	0	0	0	0	0
44	0	0	0	0	1	0
45	0	0	0	0	0	0
46	0	1	0	0	0	0

Table 4-4. Number and percentage of PCV-3 PCR positive and negative pigs during all the study period and number of PCV-3 PCR positive pigs during 1, 2, 3 and 4 or more sampling times.

Farm	PCV-3 PCR positive pigs along the study period (%)	PCV-3 PCR positive pigs at 1 sampling (%)	PCV-3 PCR positive pigs at 2 samplings (%)	PCV-3 PCR positive pigs at 3 samplings (%)	PCV-3 PCR positive pigs at ≥4 samplings (%)	Pigs PCV-3 PCR negative at all samplings (%)
A	28/34 (82.35%)	15/34 (44.12)	10/34 (29.41)	2/34 (5.88)	1/34 (2.94)	6/34 (17.65)
B	32/44 (72.73%)	14/44 (31.82)	7/44 (15.91)	3/44 (6.82)	8/44 (18.18)	12/44 (27.27)
C	22/28 (78.57%)	12/28 (42.86)	6/28 (21.43)	3/28 (10.71)	1/28 (3.57)	6/28 (21.43)
D	34/46 (73.91%)	26/46 (56.52)	5/46 (10.87)	3/46 (6.52)	0/46 (0)	12/46 (26.09)

4.3.2. Sequence alignment and phylogenetic analysis

In total, 8 PCV-3 partial sequences were finally obtained across three tested farms (Farms B, C and D) corresponding to six different animals; from two of them, sequences at two sampling points were obtained. Sequences were retrieved from four farm B pigs at 12, 18, 22 and 18 plus 22 weeks of age, respectively, one farm C animal at 25 weeks of age, and one farm D pig at 10 and 18 weeks of age. The obtained sequences comprised part of the rep protein gene (954 nucleotides). The phylogenetic tree and pairwise distance demonstrated high similarity among obtained PCV-3 partial sequences and also with the corresponding sequence fragment of the complete Spanish genome from a domestic pig available at GenBank (>99%) (Figure 4-2). In fact, most sequences obtained from farm B (4 out of 5) were identical to the one obtained from farm C, and occupied a different cluster (closer to USA and China sequences) from those coming from farm D. The two sequences from farm D were identical, coming from the same animal, and very close (99.9%) to the existing Spanish complete genome sequence from the GenBank from a domestic pig. One sequence from farm B clustered together with a German sequence, although nucleotide identity was >99% as well.

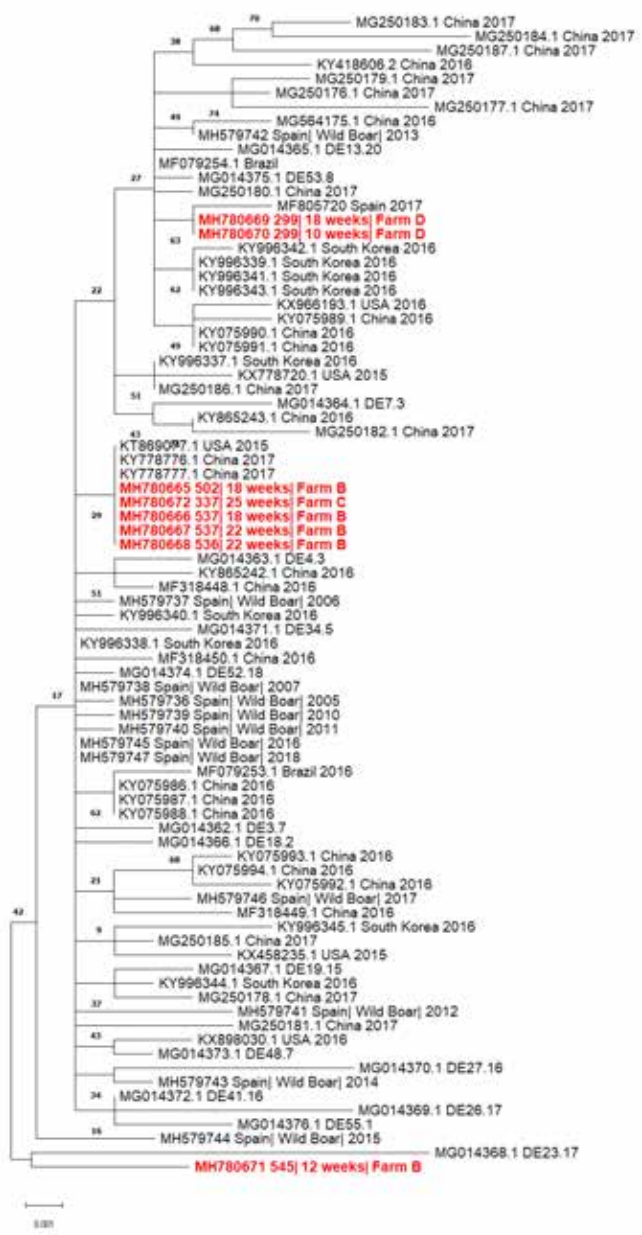

Figure 4-2. Phylogenetic tree of PCV-3 based on the partial genomes obtained from pigs longitudinally sampled and the corresponding sequences from PCV-3 full genomes available at GenBank. The phylogenetic tree was constructed using the maximum-likelihood algorithm of MEGA 7 Software with 1,000 bootstraps replicates. The obtained sequences of the present study have been colored in red.

4.4. DISCUSSION

Several epidemiological reports have detected PCV-3 genome in pigs from all production phases, associated or not with pathological disorders (Zheng et al., 2017; Zhai et al., 2017; Palinski et al., 2017; Phan et al., 2016; Chen et al., 2017). However, the lack of an existing comprehensive approach on the dynamics of infection justified to carry out specific research on longitudinally sampled animals and assess how the virus is circulating in conventional healthy farms. Moreover, already published studies testing PCV-3 frequency in different age-groups are fragmented and comparisons are not possible since information came from different sources, farms and countries. Therefore, the present study represents the first approach to investigate the PCV-3 infection dynamics in the same subset of animals.

Obtained results confirmed that this virus is apparently widespread (at least in the four selected farms), able to infect pigs at all tested ages and to cause long-term infection in few animals. In fact, there was not a particular PCV-3 infection dynamics pattern that could be inferred from the frequency of detection in the four studied farms. The higher prevalence of PCV-3 genome detection occurred at different time-points in the studied farms, which might be linked with the potential existence of maternally derived immunity or its duration.

However, while this might be the case for farms B, C and D (lower prevalence of PCV-3 infection at early ages), a different situation was found in farm A, where a moderate percentage of infected piglets was already detected at 2 weeks of age (around 26%). It is possible that such amount of PCV-3 PCR positive pigs at early ages is related with intrauterine infections, but the fact that a low-moderate percentage of pigs were found PCV-3 infected at all tested ages poses certain discussion elements on how the pig immune

system reacts against this virus. Definitively, further studies are needed to assess the circulation patterns of PCV-3 as well as to develop techniques to monitor the immune response against the virus, still lacking at present.

The most obvious comparison of PCV-3 infection dynamics is with that of PCV-2, another member of the *Circoviridae* family. In the specific case of this latter infectious agent, the virus is considered of ubiquitous nature (Segalés et al., 2005) and can be found in different age groups. However, a distinct pattern of dynamics of infection is seen for PCV-2 in non-vaccinated farms, with usual low or very low prevalence during the lactating period, loss of maternally derived immunity between 6-10 weeks of age and subsequent peak of infection during the late nursery or early fattening period (Grau-Roma et al., 2009; Larochelle et al., 2003; Sibila et al., 2004). In general, the prevalence at the peak of infection can be rather high, being close to 90-100% of infected pigs in some cases (Sibila et al., 2004; Grau-Roma et al., 2009), which is fairly different from current observations for PCV-3. An interesting point would have been the study of the infection status of sows, since at least for PCV-2 is known that infection at early ages is correlated with the percentage of infection in sows (Grau-Roma et al., 2009). Sow sera were not available for the present study, but PCV-3 has already been detected in 29% of the tested serum from sows in farms located in Poland and 47.37% in Thailand (Kedkovid et al., 2018a; Stadejek et al., 2017).

In the present study a quantitative PCR described by Franzo and colleagues (2018a) was attempted in some of the PCV-3 positive samples (data not shown). High Ct values were obtained in most of the cases, and the viral load was below the quantification limit of the PCR (10 copies of DNA/ µL). These results are in agreement with studies that detected low amount of PCV-3 DNA in serum samples (Fux et al., 2018; Stadejek et al., 2017; Zhai et al., 2017), which would suggest a subclinical infection. Moreover, this was

probably the main reason why only a few number of PCV-3 sequences were obtained.

Phylogenetic analyses and pairwise distance estimation with the eight PCV-3 partial sequences obtained throughout this study demonstrated high similarity with the corresponding sequences available at GenBank. Moreover, the sequences from the same animal (farm D) at 10 and 18 weeks of age were identical, as well as the sequence from the animal (farm B) analyzed at 18 and 22 weeks. These results would suggest possible long-lasting or persistent infections of PCV-3 in some animals with the same viral variant. In fact, this is in line with the low variability found so far with PCV-3 in comparison with PCV-2, further suggesting a much lower mutation rate of the novel virus compared with other circoviruses (Study I of this Thesis). Importantly, the potential long-lasting or persistent infections seem to be relatively frequent based on obtained results; a variable percentage ranging from 6.5% (farm D) to 25% (farm B) of analyzed pigs were PCR positive during 3 or more samplings. Long duration of infection is rather typical of ssDNA viruses infecting swine such as PCV-2 (Larochelle et al., 2003; Sibila et al., 2004) and TTSuVs (Sibila et al., 2009; Nieto et al., 2012).

Obtained partial sequences were very close each other although a broad mixing among sequences from Spain and different countries were found. However, in all cases the nucleotide identity among them was very high (>99%), suggesting that minimal variation does currently exist among PCV-3 strains. Of course, the complete genome would have been more accurate in order to distinguish potential different variants infecting the studied farms.

In summary, this is the first longitudinal study to assess the infection dynamics of PCV-3 in commercial healthy farms. Although a particular general infection dynamics pattern was not able to be ascertained, the obtained

data confirmed that PCV-3 circulated in the chosen clinically healthy farms at all tested ages and most pigs got infection during their lifetime.

CHAPTER 5

STUDY III

Porcine Circovirus 3 (PCV-3) is highly prevalent in serum and tissues and may persistently infect wild boar (*Sus scrofa scrofa*)

Published at Transboundary and Emerging Disease. 2018.
Doi: 10.1111/tbed.12988

5.1. INTRODUCTION

PCV-3 was firstly identified in the USA (Palinski *et al*., 2017; Phan et al., 2017) and the presence of viral genome has been detected subsequently in different continents such as Asia (Ku et al., 2017; Kwon et al., 2017; Shen et al., 2017), Europe (Faccini et al., 2017; Franzo et al., 2018b; Stadejek et al., 2017; Studies I and II of this Thesis) and South America. Very recently, the genome has been also detected in sera from wild boar (Franzo et al., 2018d)

Wild boar (*Sus scrofa scrofa*) are susceptible to several pathogens with potential for transmission to humans and animals (Meng et al., 2009). In fact, many viral diseases present in domestic pigs are present in boars and these animals may act as a disease reservoir (Ruiz-Fons et al., 2008). In the last decades, many European countries have experienced an increase of the wild boar population in forested and urban areas as a result of the ability of the wild boar to adapt to different environments, the high prolificacy and increased contact with humans (Castillo-Contreras et al., 2018; Fernández-Aguilar et al., 2018). In consequence, the risk of potential disease transmission between wild boar population and domestic pigs is not negligible.

The present work had a three-fold objective. First, the frequency of detection of PCV-3 in a large wild boar population of Catalonia (Spain) was tested retrospectively from 2004 to 2018. The second aim consisted of exploring the long-term dynamics of the virus in captured and re-captured wild boars. Finally, a set of captured, necropsied wild boar was used to study the tissue distribution of PCV-3.

5.2. MATERIAL AND METHODS

5.2.1. Sampling designs

5.2.1.1. Retrospective study

Serum samples (n=518) were collected from resident wild boars of 33 counties in Catalonia (North-eastern Spain) between 2004 and 2018 (Figure 5-1).

Blood was obtained by heart puncture from animals hunted during the hunting season and within the framework of the official wildlife diseases surveillance scheme or captured and euthanized for management purposes. Blood samples were centrifuged at 1,500 g for 15 min and obtained sera were stored at -20ºC until processing. The number of available sera obtained per year ranged from 3 to 18 between years 2007 and 2012, and from 30 to 88 between years 2013 and 2018 (Table 5-1). According to the age classification based on the tooth eruption patterns described by Buruaga et al. (2001), wild boars were classified as juveniles (less than 12 months), subadults (between 12 and 24 months) and adults (over 24 months). The gender of the animals was also recorded.

5.2.1.2. Longitudinal study

Nineteen boars from the metropolitan area of Barcelona (Northeastern Spain) were captured and re-captured at least two times (maximum of 6 times) for a period varying from 1 month to 1 year (Table 5-2). Blood was collected from the cranial cava vein into sterile tubes, centrifuged at 1,500g for 15 min and obtained serum stored at -20ºC until further analysis. Age-group and gender were also recorded.

Table 5-1. Number of examined wild boar and those with PCV-3 PCR positive results according to the tested year.

Year	Number of examined cases (n)	PCV-3 PCR-positive cases (n)	Percentage (%)
2004	30	3	10.00
2005	50	2	4.00
2006	46	11	23.91
2007	18	6	33.33
2008	17	5	29.41
2009	4	2	50.00
2010	10	6	60.00
2011	12	7	58.33
2012	3	3	100.0
2013	40	31	77.50
2014	50	33	66.00
2015	50	28	56.00
2016	50	4	8.00
2017	88	63	71.59
2018	50	17	34.00
Total	**518**	**221**	**42.66**

Table 5-2. PCV-3 PCR results and their respective amount of viral DNA in log_{10}/µL (in positive cases) in serum of wild boars longitudinally sampled according to the tested month and age-group at first sampling (juvenile/subadult, Ju./Sa., <2 years; Adult, >2 years). Pos= qPCR positive but under quantification limit (<10^1 PCV-3 DNA copies/µL); Neg = negative PCR result. In bold, those animals with a positive result (quantifiable or not). Underlined viral loads corresponded to those animals from which partial sequences were obtained.

Animal No	Age group at first sampling	Gender	2017									2018				No total of PCV-3 positive
			April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April	May	
1	Ju./Sa.	Female	Neg.	Neg.			Neg.	**$10^{1.3}$**						**Pos.**	Neg.		2/6
2	Ju./Sa.	Male	**$\underline{10^{2.39}}$**	**$10^{2.17}$**	**$10^{1.53}$**		**$10^{1.1}$**		**$10^{1.28}$**								5/5
3	Ju./Sa.	Male	**10^{3}**	**$10^{1.3}$**			**$\underline{10^{1.3}}$**										3/3
4	Ju./Sa.	Female	**$10^{3.06}$**				Neg.										1/2
5	Adult	Male	Neg.													**$10^{1.3}$**	1/2
6	Ju./Sa.	Female	**$\underline{10^{1.62}}$/ $\underline{10^{2}}$†**	**$\underline{10^{1.3}}$**													3/3
7	Adult	Female	Neg.													**$10^{1.3}$**	1/2
8	Adult	Female	Neg.	Neg.													0/2
9	Ju./Sa.	Male		Neg.‡													0/1
10	Adult	Female		Neg.	Neg.	Neg.		Neg.	Neg.							Neg.	0/6
11	Adult	Female		Neg.				Neg.									0/2

Table 5-2 (continuation). PCV-3 PCR results and their respective amount of viral DNA in log_{10}/µL (in positive cases) in serum of wild boars longitudinally sampled according to the tested month and age-group at first sampling (juvenile/subadult, Ju./Sa., <2 years; Adult, >2 years). Pos= qPCR positive but under quantification limit (<10^1 PCV-3 DNA copies/µL); Neg = negative PCR result. In bold, those animals with a positive result (quantifiable or not). Underlined viral loads corresponded to those animals from which partial sequences were obtained.

Animal No	Age group at first sampling	Gender	2017									2018					No total of PCV-3 positive
			April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April	May	
12	Ju./Sa.	Female			**$10^{1.8}$**	**$10^{1.22}$**		Neg.							Neg.		2/4
13	Ju./Sa.	Female					Neg.	Neg.			Neg.	Neg.				Neg.	0/5
14	Ju./Sa.	Male					**$10^{3.1}$**	**$10^{2,75}$**			**$10^{1.56}$**	**$10^{1.56}$**					4/4
15	Adult	Female					Neg.								Neg.		0/2
16	Ju./Sa.	Female					Neg.	Neg.								Neg.	0/3
17	Ju./Sa.	Male					Neg.		Neg.								0/2
18	Ju./Sa.	Female										**Pos.**				**Pos.**	2/2
19	Ju./Sa.	Male										Neg.			Neg.		0/2

† Animal longitudinally sampled twice in April, 2017. PCV-3-PCR was positive in both samplings.‡ Animal longitudinally sampled twice in May, 2017. PCV-3 PCR was negative in both tested samples.

5.2.1.3. Study on tissues, feces and serum

Thirty-five wild boar captured and euthanized for management purposes in Catalonia (North-eastern Spain) were selected for this study. Sera samples from 28 out of 35 selected animals were available as well as 33 faecal samples. Eight different tissue types were collected. Tonsil, liver, lung, spleen, kidney, and brain were analysed from all boars selected for this study, while submandibular lymph nodes were only available from 30 wild boar (Table 5-3).

5.2.2. DNA extraction

DNA was extracted from 200 µL of serum using MagMAx™ Pathogen RNA/DNA Kit (Applied Biosystems®) according to the manufacturer's protocol. For faecal samples, DNA was extracted from 200 mg of feces with QIAmp DNA Stool Mini Kit (QIAGEN®). Finally, approximately one cm^3 of tissues (corresponding to 180-200 mg) were diluted in 1 mL of sterile Phosphatase-buffered saline (PBS, pH 7.4), and then homogenized with the TissueLyser II (QIAGEN®) for 30 min at 14,000 rpm DNA from the homogenized tissue was extracted according to the same protocol described for serum samples.

Table 5-3. Number of tested samples (serum and tissues) and their PCV-3 PCR result and percentage of positives.

Samples	No of tested samples (n)	No of PCV-3 PCR Positive samples	Percentage (%)
Sera	28	5	17.86[a]
Feces	33	3	9.09[a]
Brain	35	10	28.57[a]
Heart	01	1	100[a]
Kidney	35	10	28.57[a]
Liver	35	19	54.29[b]
Lung	35	20	57.14[b]
Sub. LN	30	9	30[a]
Spleen	35	19	54.29[b]
Tonsil	35	15	42.86[b]

Different letters in superscript mean statistically significant differences ($p<0.05$) among different sample types tested. Sub. LN: Submandibular Lymph Node

5.2.3. Conventional and quantitative PCRs to detect PCV-3

All primers and probes used in this study are included in Table 5-4.

To detect the presence of PCV-3 DNA in the tested samples, a conventional PCR assay was performed based on a previous protocol described by Franzo et al. (2018a), with slight modifications. Three µL of extracted DNA

was added to a PCR mix and amplified using the described thermal protocol. The reaction was carried out in a final volume of 50 µL mixture containing 5x PCR Buffer, 10 pmol of dNTPs, 10 pmol of each primer, 1 Units of DNA polymerase Platinum™ SuperFi™ (Invitrogen™) and water to bring the final volume up to 50 µL. The PCR products were checked on 1.2% TAE agarose gel.

A previously published quantitative PCR (qPCR) protocol (Franzo et al., 2018a) with slight modifications, was performed on all positive samples tested by conventional PCR from different tissue types and animals longitudinally sampled. Reactions were carried out with an Applied Biosystems® 7500 Real-Time machine. Briefly, 2 µL of extracted DNA was added to a standard mixture containing 1x Quantitect Probe PCR mix (QIAGEN®), 0.6 pmol of each primer and 0.3 pmol probe, 1 pg of the internal control (IC) plasmid vector pAcGFP1-1 (Takara- Clontech®), 0.4 pmol and 0.2 pmol of IC primers and probe, respectively, and sterile water to bring the final volume up to 20 µL. The thermal protocol included 95ºC for 15 min followed by 45 cycles of 95ºC for 10 sec and 60ºC for 1 min. Viral concentrations were expressed as mean of $\log^{10}$ PCV-3 genome copies/µL and the limit of quantification were considered at least 10 copies of DNA per µL.

Table 5-4. Primers and probes implemented in the conventional PCR, quantitative PCR (qPCR) and PCRs for the partial/complete genome sequencing used in this study.

Primers and/or probes	Start Position	Sequence 5'-3'	Assay	Reference
PCV3$_{233}$F	233	5'-AAAGCCCGAAACACAGGTGGTGT-3'	Conventional PCR	Franzo et al., 2018a
PCV3$_{718}$R	718	5'-TTTTCCCGCATCCTGGAGGACCAAT-3'		
PCV3$_{353}$F	353	5'-TGACGGAGACGTCGGGAAAT-3'	qPCR	Franzo et al., 2018a
PCV3$_{465}$R	465	5'-CGGTTTACCCAACCCCATCA-3'		
Probe_qPCR	418	5'-FAM-GGGCGGGGTTTGCGTGATTT-BHQ1-3'	Viral detection	
PCV3$_{506}$F_IC	506	5'-TCCTGGGCAATAAGATGGAG-3'	Internal control	This Thesis
PCV3$_{661}$R_IC	661	5'-TGGGGGTATTCTGCTGGTAG-3'		
Probe_IC	528	5'-VIC-CCACTACAACGCCCATG-MGBNFQ-3'		

Table 5-4 (continuation). Primers and probes implemented in the conventional PCR, quantitative PCR (qPCR) and PCRs for the partial/complete genome sequencing used in this study.

Primers and/or probes	Start Position	Sequence 5'-3'	Assay	Reference
PCV74F	74	5'-CACCGTGTGAGTGGATATAC-3'	Conventional PCR-partial and complete genomes	Fux et al., 2018
PCV31444R	1444	5'-CACCCCAACGCAATAATTGTA-3'		
PCV31137F	1137	5'-TTGGGGTGGGGGTATTTATT-3'		
PCV31561R	1561	5'-ACACAGCCGTTACTTCAC-3'		
PCV31427F	1427	5'-AGTGCTCCCCATTGAACG-3'		
PCV3433R	433	5'-CGACCAAATCCGGGTAAGC-3'		

5.2.4. PCV-3 sequencing and phylogenetic studies

For genome sequencing, 3 µL of the extracted DNA was added to the PCR mixture described above for the conventional PCV-3 PCR, using the thermal protocol and three specific primer pairs (Table 4) able to detect three amplicons described by Fux et al. (2018). The PCV-3 PCR-positive samples were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) according to the manufacturer's protocols and the quality and quantity of genomic DNA was analysed with BioDrop DUO (BioDrop Ltd).

The selected samples were submitted to Sanger-sequencing, which was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit, following the manufacturer's protocol at the Genomic and Bioinformatics Service of the *Universitat Autònoma de Barcelona* (Barcelona, Spain). The sequencing reactions were analysed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®)**.**

Sequences and chromatograms were manually explored to trim bad-quality bases with BioEdit 7.2 (Hall, 1999). The assembly of the consensus sequences extracted from different fragments was done with DNASTAR Lasergene software (Burland, 1999). Both complete and partial genomes obtained were aligned using Clustal Omega (Thompson et al., 1997). A collection of reference sequences available at the GenBank were included in the analysis (Table 5-5). Pairwise identity matrices were obtained using BioEdit software. Phylogenetic analysis was performed with MEGA software vs 7 (Kumar et al., 2016) with the Maximum-Likelihood (ML) method based on the best predicted-model (lowest BIC score), i.e. Tamura-Nei substitution model (Tamura & Nei., 1993). Robustness of the ML tree was evaluated by analysis with 1,000 bootstrap replicates.

In total, 24 samples were selected to obtain partial and complete PCV-3 genome sequences. Twelve complete sequences were obtained from animals corresponding to different years of the retrospective study. Partial sequences were obtained from 5 animals longitudinally sampled in two and/or three different time-points (n=9) and from tissue samples of one wild boar (n=3). The sequences obtained throughout this study are available at the GenBank (accession numbers MH579736–MH579747 for complete sequences of the retrospective study, MH751283-MH751287 and MH751293-MH751296 for partial sequences of the longitudinal study, and MH751289- MH751291 for partial sequences from tissues).

Table 5-5. List of sequences included in the analysis according to the country and collection date

Genbank ID	Collection country	Collection date
MF079254	Brazil	2016
MF079253	Brazil	2016
KY075990	China	2016
KY075991	China	2016
KY075989	China	2016
KY865243	China	2016
MG250182	China	2017
MG250180	China	2017
MG564175	China	2016
KY418606	China	2016
MG250179	China	2016
MG250176	China	2016
MG250177	China	2016
MG250187	China	2017
MG250183	China	2017
MG250184	China	2017
MG250185	China	2017
KY075992	China	2016
KY075993	China	2016
KY075994	China	2016
MG250186	China	2017
KY865242	China	2016
KY778776	China	2017
KY778777	China	2017
MG250178	China	2017
KY996340	China	2016
KY075986	China	2016
KY075987	China	2016
KY075988	China	2016
MF318448	China	2016
MF318449	China	2016

Table 5-5 (continuation). List of sequences included in the analysis according to the country and collection date

GenBank ID	Collection country	Collection date
MF318450	China	2016
MG014365	Germany	2015
MG014375	Germany	2015
MG014364	Germany	2015
MG014373	Germany	2015
MG014370	Germany	2015
MG014369	Germany	2015
MG014376	Germany	2015
MG014362	Germany	2015
MG014372	Germany	2015
MG014368	Germany	2015
MG250181	Germany	2015
MG014367	Germany	2015
MG014366	Germany	2015
MG014363	Germany	2015
MG014371	Germany	2015
MG014374	Germany	2015
KY996341	South Korea	2016
KY996343	South Korea	2016
KY996339	South Korea	2016
KY996342	South Korea	2016
KY996344	South Korea	2016
KY996345	South Korea	2016
KY996337	South Korea	2016
KY996338	South Korea	2016
MF805720	Spain	2017
KX966193	USA	2016
KX898030	USA	2016
KX458235	USA	2015
KX778720	USA	2015
KT869077	USA	2015

5.2.4. Statistical analyses

Statistical analyses were performed using the R software (http://www.r-project.org/). Shapiro Wilk's test was used to evaluate the normality of the distribution of the quantitative variables.

Differences over the years were analysed by the Pearson's Chi-squared test (χ^2) in the retrospective study; for such comparison, a subdivision of five groups containing three tested years each one was created. To assess the association between age-groups, the same test was implemented. To test differences between gender and the PCV-3 PCR positivity between the tested counties, the Fisher's exact test was performed.

For the purpose to test the differences between the PCV-3 PCR positivity frequency in tissues, the Pearson's Chi-squared test (χ^2) was carried out. P-values lower than 0.05 were considered to be statistically significant.

5.3. RESULTS

5.3.1. PCV-3 detection by PCR and quantification by qPCR

5.3.1.1. Retrospective study

PCV-3 was found in wild boar of all counties studied (Figure 5-1). No significant association was found between county abundance of wild boar and frequency of PCV-3 detection (Figure 5-2).

The first PCV-3 PCR-positive sample was detected in the first year of testing (2004) and subsequently PCV-3 genome was found in all examined years (Table 5-1). In total, 221 out of 518 (42.66%) serum samples were PCR-positive for PCV-3 and the percentage of PCV-3 positivity ranged from 4% (2 out of 50) in 2005 to 100% in 2012 (in which only 3 samples were tested).

Significant differences were observed across the tested years, with higher frequencies of PCV-3 PCR-positivity found in both periods 2013-15 and 2016-18 ($p<0.05$) compared to the previous ones. The frequency of PCV-3 genome detection in wild boar was significantly higher in adults than in was subadults or juveniles (adults 47.5%, 152 out of 320; subadults 25.27%, 23 out of 91; juveniles 8.69%, 2 out of 23) ($p<0.05$). PCV-3 positivity was found in 111 out of 253 tested females and in 90 out of 212 males, no significant difference of PCV-3 frequency was found between genders.

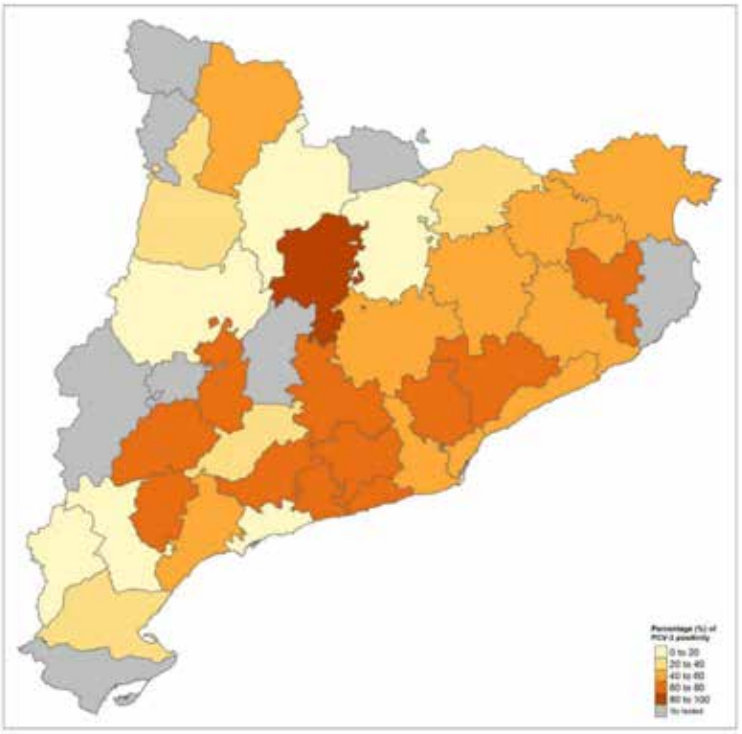

Figure 5-1. Distribution of the tested wild boar in Catalonia (Spain) of PCV-3 PCR positive animals according to each county. The darker the colour intensity, the higher the PCV-3 frequency detection by PCR.

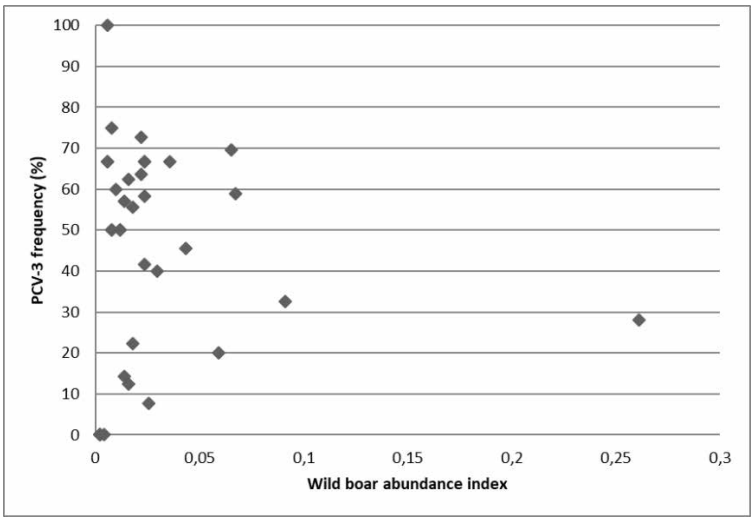

Figure 5-2. Wild boar abundance in the different counties according to PCV-3 frequency of detection by PCR. The wild boar abundance is based in the relative density index (RDI). RDI is calculated by dividing the number of wild boars hunted between the geographical surface of hunting and the number of hunting beaters.

5.3.1.2. Longitudinal study

PCR and qPCR results are summarized in Table 5-2.

PCV-3 PCR positivity was found in 10 out of 19 longitudinally tested wild boar (52.63%). Five of these animals (i.e. No. 2, 3, 6, 14 and 18) were PCV-3 PCR-positive at all samplings performed throughout the study period. Globally, 3 animals were positive for PCV-3 in only one sampling (No. 4, 5 and 7) while the rest of wild boar were positive in at least two samplings separated by 2 (n=2, No. 6 and 12), 5 (n=2, No. 3 and 18), 6 (n=1, No. 14) or 7 months (n=2, No. 1 and 2).

The amount of PCV-3 DNA obtained through qPCR was low to moderate, ranging from $10^{1.1}$ to $10^{3.1}$ copies of DNA/µL serum. Three out of 24 qPCR- positive samples (from wild boar No. 1 and 8) were not within the

limits of quantification (less than 10 copies of DNA/µL). PCV-3 PCR positivity was found in 8 out of 13 (61. 54%) tested juveniles/subadults and in 2 out of 6 (33.33%) adults. PCV-3 was found in 50% (6 out of 12) of tested females and in 57. 14% (4 out of 7) of males. No significant differences were detected in PCV-3 PCR positivity between the tested age-groups and genders.

5.3.1.3. Study on tissues, feces and serum

The frequency of PCV-3 DNA detection in tissues, serum and feces is displayed in Table 3.

PCV-3 DNA was found in all tested sample types. In total, 32 out of 35 (91.43%) wild boar were positive for PCV-3 in at least one tested sample. The median of the amount of DNA per sample type per µg ranged from $10^{2.26}$ to $10^{4.18}$ copies of DNA/µL in tonsil and submandibular lymph node, respectively. Only 30 samples (out of 111 positives by PCR) were quantifiable, with more than 10 copies of DNA/µL. Figure 5-3 shows the load found in the different tested tissues from wild boar. Significant differences in PCV-3 frequency were detected in tonsil (p=0.0334), lung (p=0.0017), liver (p=0.0039) and spleen (p=0.0039) compared to the other tissue types.

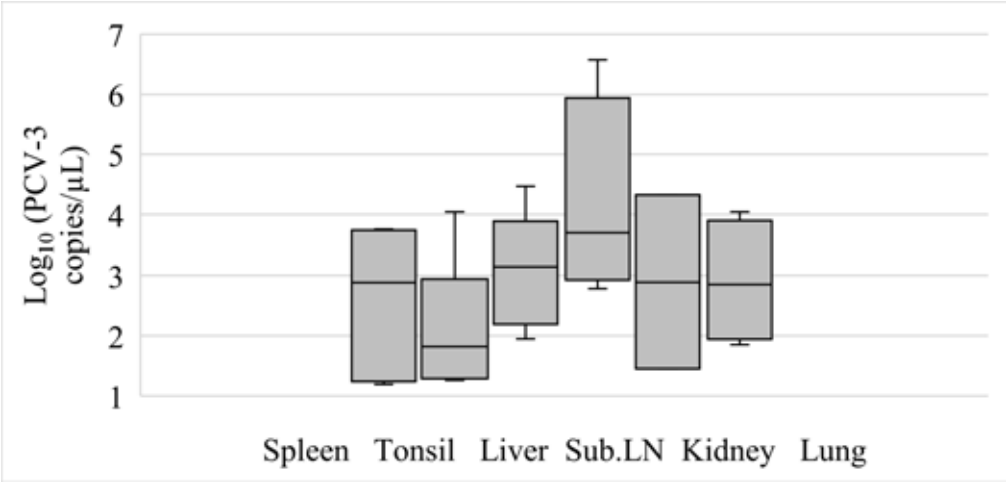

Figure 5-3. Boxplot reporting the viral load found in the different tested tissues from wild boar; line within the boxplot represents the median of viral load. Samples with more than 10 copies of DNA/µL were considered quantifiable based on the used qPCR methodology(Franzo et al, 2018b). Sub.LN: Submandibular lymph node.

5.3.2. Phylogenetic analysis

The PCV-3 complete nucleotide sequence of 12 animals from the retrospective study were obtained and compared through phylogenetic analysis. Different complete sequences available at GenBank both from Europe, including one from Spain, and worldwide were used for comparison. The full genome sequences from wild boar from different years clustered in the same group together with few strains of domestic pigs from China and Germany (Figure 5-4). The pairwise distance analysis showed a minimum of 98% of identity among the samples from wild boar and between the PCV-3 full-genome sequences from domestic pigs. Wild boar partial cap sequences available from GenBank (Franzo et al., 2018d) were translated and used for comparison with obtained full sequences, showing as well >98% nucleotide identity and clustering together.

The similarity of partial nucleotide sequences obtained from the other two studies was compared to determine potential coinfection with different strains in the same animal (tissue/feces/serum study) or infection with different strains at different time points (longitudinal study). When partial sequences obtained from different tissues of the same wild boar were compared, they demonstrated to be highly similar (>98.4 %) supporting the idea of being likely the same strain. Globally, high similarity was also found between sequences of the longitudinal study (>96.6%); however, when comparing those coming from the same animal, nucleotide identity was >99%, suggesting that animals were infected by the same strain for a long period of time.

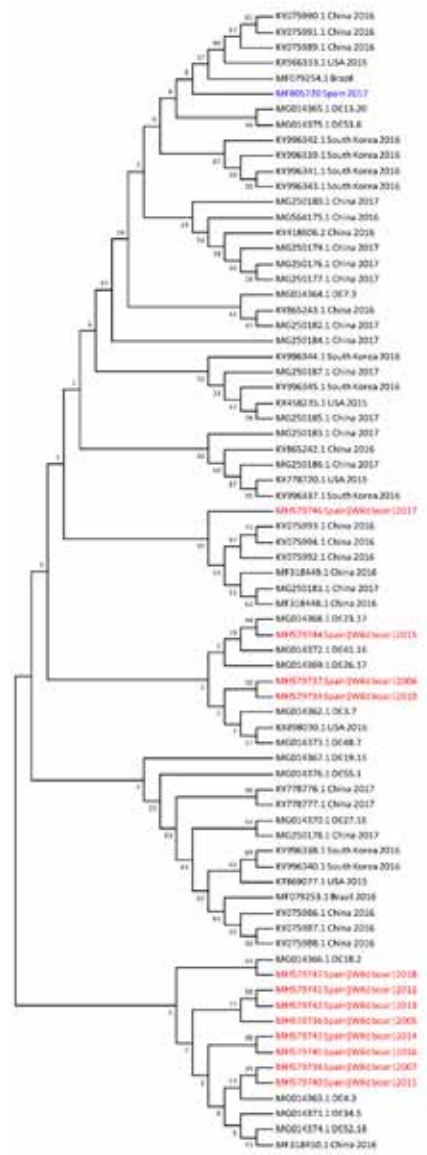

Figure 5-4. Phylogenetic tree based on the complete genomes of PCV-3 Spanish strains from wild boar and PCV-3 freely available sequences at GenBank. The phylogenetic tree was constructed using the maximum-likelihood algorithm of MEGA 7 software with 1.000 bootstrap replicates. Bootstrap values supporting the clusters were represented. Spanish sequences obtained in the present study and Spanish sequence from domestic pigs have been colored in red and blue, respectively.

5.4. DISCUSSION

This study represents the first report evaluating the PCV-3 frequency in retrospective serum samples from wild boars. Moreover, the assessment of the dynamics of the virus in captured and re-captured boars and the tissue distribution of PCV-3 genome in this species was also investigated for the first time. Importantly, all three independent studies confirmed that wild boar are susceptible to PCV-3 infection as previously indicated (Franzo et al., 2018d). Such susceptibility was probably expected since several reports indicates that most of the pathogens infecting domestic pigs are also present in the wild boar population (Ruiz-Fons et al., 2008). This scenario is paralleled with another circovirus species infecting swine, *Porcine Circovirus* 2 (PCV-2), which has been shown to be ubiquitous in the wild boar population worldwide (Ruiz-Fons et al., 2008). In the particular case of PCV-2, it has also been demonstrated that wild boar can develop the PCV-2 systemic-disease, characterized by weight loss, wasting, diarrhoea, weakness, jaundice, lymphadenopathy and respiratory problems without response to antibiotic treatments (Lipej et al., 2007). No evidence of disease caused by PCV-3 in wild boar is so far available.

According to the present study, PCV-3 has been circulating in the Spanish wild boar population at least since 2004, the earliest evidence of infection found in this animal species. This result is in line with recently published reports confirming the circulation of PCV-3 in domestic pigs since 1990s (Ye et al., 2018). Although the PCV-3 frequency reported in pigs vary greatly, ranging from 10 to 75% in serum samples, the overall results obtained in this study suggest that, apparently, the PCV-3 frequency in boars is higher than that found in domestic pigs from European countries like Spain (11.46%), Italy (18.18%), Poland (25%) and Denmark (30%) (Faccini et al., 2017; Franzo et al., 2018a; Stadejek et al., 2017; Studies I and II of this Thesis). Therefore,

obtained data may suggest a potential reservoir role of the wild boar in respect PCV-3 infection.

Noteworthy, the frequency of infected wild boar between years 2013 and 2018 (53.66%) was higher than that between 2004 and 2012 (23.68%). Although it may be speculated that this was due to infection dissemination into a potential naïve population of animals, it cannot be ruled out the simple effect of highly efficient contacts between susceptible and infected wild boar due to the significant increase of wild boar densities in the studied geographical area during last decade (Massej et al. 2015).

PCV-3 evidence of infection was observed over a large period of time in few animals (5-7 months). On one hand, this may suggest a persistent or long-lasting viral infection, similar to what has been described for hepatitis E virus (HEV) and PCV-2 in the wild boar (Boadella et al., 2011). On the other hand, an infection with subsequent reinfection could also be a possibility. However, the highly similar nucleotide identity among PCV-3 sequences available in this study and the GenBank database (for both wild boar and domestic pigs) prevents the confirmation of this latter hypothesis. For HEV, the longer the period of viremia, the higher the likelihood for the role of wild boar as reservoir (Schlosser et al., 2015). For example, such role as reservoir for persistently infecting viruses like bovine viral diarrhoea, Aujeszky's disease virus and classical swine fever virus is well known in the wild boar (Ruiz-Fons et al., 2008). In consequence, the apparent long-lasting infection described in the present study for PCV-3 would also reinforce the notion of wild species as potential reservoir for the domestic pig. However, it is still too early to confirm such reservoir status for PCV-3, and further studies will be needed to elucidate the epidemiological role of this virus infection in wild boar.

Interestingly, some significant differences in the PCV-3 frequency were observed by age; compiled data in the retrospective study showed that adult animals were more often PCV-3 PCR positive than juveniles or subadults. However, this difference was not observed between age-groups in the longitudinal study, probably due to a much lower sample size. If data from both studies are taken together, adult wild boar seem to be viremic to a higher frequency. This is in line with the so far only published article on PCV-3 in wild boar, where a lower prevalence in juveniles was also detected (Franzo et al., 2018d). Such age-group comparison was also performed for PCV-2 in wild boar by means of serology, but again no significant differences were observed (Vicente et al., 2004). Similarly, available data on domestic pigs do not point out to a potential higher frequency of PCV-3 detection in any specific age group (Kwon et al., 2017; Stadejek et al., 2017; Studies I and II of this Thesis).

The pathogenic role of PCV-3 infection is still unclear (Franzo et al., 2018a; Sun et al., 2018). Detection of viral genome in serum indicates a systemic infection, but there are still no clues on the main target organs or cell tropism. As an exploratory approach, different tissue samples and feces in addition of serum were tested in a subset of wild boar. PCV-3 genome was detected in all tested tissue types as well as in feces to a higher frequency and viral load when compared with serum. In consequence, it seems evident that detection of PCV-3 in serum underestimates significantly the percentage of infected wild boar, fact that also happens to a number of ssDNA viruses in the domestic pig (Nieto et al., 2013; Calsamiglia et al., 2002). Although obtained results are preliminary, percentage of truly infected wild boar surpassed 90%, further suggesting potential long-lasting infections and putative defective immune responses (not able to neutralize and clear the virus from the organism).

Also, significant differences in the PCV-3 frequency between the tested tissues showed that the most useful tissues for PCV-3 detection were tonsil, liver, spleen and lung, which may account for target organs for PCV-3 replication. Moreover, although positivity was lower than in other tissues, the submandibular lymph node offered the highest viral loads. Different reports have been shown the presence of the virus in different lymphoid tissues with a higher frequency in lung and lymph nodes (Fan et al., 2017; Fu et al., 2017). In all cases, and although it implies underestimating the real frequency of infection, serum is still the most appropriate sample for epidemiological studies. The amount of PCV-3 DNA in tissues was considered low to moderate, in agreement with several studies which detected low viral load in the analysed samples of domestic pigs (Fux et al., 2018; Stadejek et al., 2017; Zhai et al., 2017). In all cases, these low viral loads suggest that wild boar, similar to domestic pigs, might be subclinically infected with this virus. At least for PCV-2, an association between the viral load and the severity of lesions has been described, suggesting that high amount of DNA is a major feature of pigs affected by PCV-2 systemic disease (Olvera et al., 2004). Moreover, in the few described cases of PCV-2 systemic disease in wild boar, high loads of PCV-2 were also found in tissues (Ellis et al., 2003; Vicente et al., 2004).

A total of 24 partial or complete sequences of PCV-3 corresponding to the three different studies were obtained. The phylogenetic analysis of these sequences indicated a close distance between them and with other PCV-3 genomes available at the GenBank from both domestic pig and wild boar. The Spanish wild boar sequences were located in the same cluster that others from domestic pigs from Germany and China. Interestingly, the only complete genome from domestic pigs from Spain is located away of wild boar sequences.

These data also reinforcing the notion that PCV-3 does not show independent molecular evolution in the particular areas of the world where it has been detected to date (Study I of this Thesis).

In conclusion, the present study further demonstrates that wild boars are susceptible to PCV-3 infection and confirms the virus circulation at least since 2004 with a relatively high frequency. According to the results, PCV-3 can be detected over a long period of time, suggesting long-lasting infections do occur in wild boar. In addition, PCV-3 was detected in all tested tissue sample types and feces, being the most frequently positive tissues tonsil, lung, liver and spleen. Globally, high nucleotide identity was found in all PCV-3 sequences obtained from wild boar

CHAPTER 6

GENERAL DISCUSSION

Porcine circoviruses (PCVs) are ubiquitous viruses, spread worldwide in the domestic pig population. On one hand, PCV-1 is a persistently contaminated agent of CCL-33 PK-15 cell line, considered a non-pathogenic virus (Tischer et al., 1986). On the other hand, PCV-2 is associated with several clinical manifestations collectively named PCVDs, which caused and still cause huge economic losses in the pig industry (Kekarainen and Segalés, 2015; Madson and Opriessnig, 2011). Very recently, a third species of PCVs, named PCV-3, has been discovered through NGS techniques in samples from animals affected by reproductive failure and cardiac and multisystemic inflammation (Palinski et al., 2017; Phan et al., 2016). At the beginning of this Thesis, very limited information was available for this novel virus, consisting in only two published works. Subsequently, and concomitantly to this Thesis research work, several investigations have been demonstrated the presence of this virus in different countries, diverse sample types as well as in pigs from different production phases, either associated or not with clinical and/or pathological conditions (Franzo et al., 2018b; Kedkovid et al., 2018a; Zhai et al., 2017; Zheng et al., 2017). In fact, nowadays, it is still unclear whether PCV-3 is responsible for a particular swine disease and if exist causality of the virus with the lesions reported above. Although at the beginning of the present Thesis the knowledge on this new PCV was minimal, the interest in PCV-3 was significant within the veterinary and scientific communities. This was especially due to the fact that one of the PCV species, PCV-2, is a devasting pathogen in the pig husbandry that have been in the pig population worldwide for many decades before it was recognized as a cause of overt disease. Therefore, with the purpose of filling knowledge gaps in PCV-3, the major aim of this Thesis was to investigate the epidemiology of this virus in two *Suidae* species, the domestic pig and the wild boar.

To achieve the proposed goals, molecular tools based on PCR and Sanger sequencing were used. As PCV-3 is a newly discovered virus, there is still a very limited diagnostic toolbox available. The retrieval of the viral sequence from the first study (Palinski et al., 2017) prompted the development of molecular techniques, mainly used for research and limitedly in diagnostic laboratories; most of these techniques are hardly standardized and still needed refinement. PCV-3 isolation has been attempted in PK-15 and ST cells, without successful results to date (Faccini et al., 2017; Palinski et al., 2017). Very recently, a PCV-3 Cap-specific monoclonal antibody and a recombinant Cap protein have been developed (Li et al., 2018a), opening the avenue for the potential development of techniques such as immunohistochemistry and antibody detection assays. In consequence, there is still no information about immunity and pathogenesis of PCV-3 infection.

Although PCV-3 has recently been described, there was no information on how long the virus has been circulating in the pig population. Palinski and colleagues (2017) conducted a brief study in paraffin fixed tissues from 2010 to 2016 in North America. Results showed high percentage of PCR positivity in these samples, suggesting that the virus emerged before the year of its discovery. In consequence, a relevant aim addressed in this Thesis was to investigate such scenario. In *Chapter 3*, a retrospective study in serum samples from domestic pigs throughout a 22-year period was performed. PCV-3 was already present in the Spanish pig population at least since the first tested year, 1996. Two works carried out almost simultaneously to this study, one from China and another from Sweden, revealed similar results: the first detection of PCV-3 in swine samples was dated in 1996 (Sun et al., 2018) and 1993 (Ye et al., 2018), respectively. In conclusion, PCV-3 has been systematically detected in some of the oldest samples so far tested, suggesting that this is not a new virus, although went unnoticed for decades. Of course, this poses a big question

mark regarding its virulence and association with disease. However, such results cannot be assumed as a proof of non-pathogenicity, especially when mirroring another closely-related circovirus, PCV-2. Although this latter virus was initially detected in association with disease, retrospective studies showed evidence of pig infection a number of decades before. These works revealed that the virus was circulating much before the first report in 1991 (Clark, 1996; Harding, 1996): at least since 1962 in Germany (Jacobsen et al., 2009), 1973 in Mexico (Ramírez-Mendoza et al., 2009), 1978 in Brazil (da Silva et al., 2011) and 1985 in Spain (Rodríguez-Arrioja et al., 2003). In fact, in most of these investigations, evidence of PCV-2 infection coincided with the very first investigated year, suggesting that PCV-2 might even be an older circulating virus. In addition, in *Chapter 5*, serum samples from wild boar were also tested retrospectively during a 14-year period; as expected, PCV-3 was detected in the boar population, in agreement with a previous study conducted in Italy (Franzo et al., 2018d). Importantly, the virus was present in wild boar since the first tested year as well (2004). Overall, obtained data confirmed that PCV-3 is not a new virus and has been circulating for a fairly, non-determined long time in swine and wild boar populations. In support of these findings, bioinformatics analyses have estimated the most common ancestor (TMRCA) of PCV-3 to be originated approximately in 1966 (Fu et al., 2017; Saraiva et al., 2018). In the same way as PCV-3, evolutionary analyses revealed that PCV-2 TMRCA probably emerged around 100 years ago (Firth et al., 2009).

To establish whether exist an association between PCV-3 positivity and disease, the animals used for the retrospective analysis were classified according clinical/pathological disorders; a group of apparently healthy animals were also included as negative control (*Chapter 3*). No disease association pattern was observed among tested animals. However, it must be indicated that this study was designed with the main objective of detecting

PCV-3 retrospectively, and not to study specifically relationship with clinical problems. In fact, current literature has already reported the presence of PCV-3 in animals with different clinical pictures: one of them was performed with samples from diseased animals with respiratory conditions or diarrheal signs compared with apparently healthy animals (Zhai et al., 2017) and the other one performed in natural stillborn piglets (Zheng et al., 2017). In these studies, diseased animals showed higher prevalence of the viral infection compared to the healthy ones. Therefore, further studies should be done to ascertain a potential association with disease manifestation.

Co-infection of PCV-3 with both PCV-2 and PRRSV was found in the retrospective study (*Chapter 3*), in line with other reports (Kedkovid et al., 2018b; Ku et al., 2017; Sun et al., 2018; Zhao et al., 2018). In fact, this was expected since both well-known pathogens are widespread in the pig population (Franzo et al., 2015c; Madec et al., 2008; Shi et al., 2013). Noteworthy, it is known that both PCV-2 and PRRSV are able to affect the immune system and, therefore, co-infections with these viruses and/or other infectious agents are not unusual (Dekkers et al., 2017; Grau-Roma et al., 2011). Further investigations are needed to determine whether PCV-3 might act as one of these secondary agents that may up-regulate its replication once pigs are immunosuppressed or immunomodulated, or whether the frequency of co-infection is independent of the immune system affection.

In *Chapter 3*, no differences in terms of PCV-3 PCR-positive frequency among production phases were found. These results are not in accordance with previous works that detected variability according to age groups, with higher prevalence in weaned pigs (Fux et al., 2018; Kwon et al., 2017; Stadejek et al., 2017). The second study of this Thesis (*Chapter* 4) intended to better clarify such scenario by means of assessing the dynamics of PCV-3 infection in four sets of longitudinally monitored pigs. Such approach improved previous

publications in which their designs did not include the same animals at different ages but different pigs from fairly limited age-groups. Results obtained from the present Thesis suggest a lack of a traditional infection dynamics pattern, in which a significant percentage of pigs would get infection at a given time point. PCV-3 infected pigs were found at all age-groups in all farms, and the frequency of infection was not clearly dominant at any age. Such finding was surprising, since previous experiences with other circoviruses, especially PCV-2, indicate a relatively clear infection pattern. Pigs infected by PCV-2, with or without a clinical picture of PCV-2-SD, are regularly found between five and 12 weeks of age, and rarely in animals at the lactation phase (Allan and Ellis, 2000; Larochelle et al., 2003; Sibila et al., 2004). This is explained by the fact that colostrum antibodies are protective against infection and then decline during the lactation and weaning phases; once maternally derived antibodies waned, an active infection is followed by active seroconversion (Larochelle et al., 2003; Rodriguez-Arrioja et al., 2002; Sibila et al., 2004). This seroconversion usually occurs between 9 and 15 weeks of age and the antibodies may last until 28 weeks of age at least (Grau-Roma et al., 2009; Larochelle et al., 2003; McIntosh et al., 2006; Rodríguez-Arrioja et al., 2003). Regrettably, information about infection in sows, maternally derived immunity and how protective the immunity might be against PCV-3 is completely lacking at the moment. It is known that PCV-3 can be found in colostrum (Kedkovid et al., 2018a), implying the possibility of vertical transmission (sow to piglet) and emphasizing the potential importance of early infections. Again, available information regarding these issues on PCV-3 is still to be generated.

In addition, in the *Chapter 5*, a set of captured and re-captured wild boar were also longitudinally tested in a variable period of their life. Unfortunately, the limited number of animals does not allow establishing a

proper infection dynamics pattern of PCV-3 in this species. However, results indicated that a number of wild boar developed a long-lasting infection (potential persistent infection), since the virus could be detected during a period of at least 5-7 months in few animals. Susceptibility of wild boar to PCV-3 was not a surprise, since this species shows susceptibility to several pathogens that affect humans and animals (Meng et al., 2009), including PCV-2; moreover, the wild boar can also develop PCV-2-SD (Lipej et al., 2007). Although for a number of pathogens the domestic pig is considered the source of infection for wild boar (Ruiz-Fons et al., 2008), *Chapter 5* opens the discussion about the wild boar acting as a PCV-3 reservoir for domestic pigs. Taking into account the potential large period of infection observed in some animals and even a higher overall prevalence when compared with domestic pigs (*Chapter 4*), such potential reservoir role deserves further investigations.

PCV-3 organic distribution has not been investigated in a comprehensive manner, although some studies have given some insights in the domestic pig (Xu et al., 2018; Zhai et al., 2017). In order to gain new data on PCV-3 distribution, serum samples, feces and a set of tissues were tested in the wild boar (*Chapter 5*). Moreover, the quantification of PCV-3 positive samples was also performed. Presence of viral genome was determined in all tested sample types. Liver, spleen and lung were the most useful tissues to detect PCV-3 in therms of frequency, but the submandibular lymph node showed the highest viral load. Viral load is an important concept for a number of viruses; for example, the higher the PCV-2 load, the severe the lesions found in PCV-2-SD (Olvera et al., 2004). Such correlation has not been established for PCV-3, but the data obtained so far in *Chapter 5* indicated very low viral loads, fitting well with a subclinical infection scenario. In *Chapters 3* and *4*, the quantification of genome was performed as an exploratory approach in PCV-3 positive samples. Data compiled in all studies of this Thesis showed

moderate to high Ct values. Moreover, in some cases the detection was possible by standard PCR, but the viral load was below the limit of quantification of the qPCR, further emphasizing the subclinical nature of the infection. Therefore, further studies are necessary to potentially elucidate the correlation of PCV-3 load and potential disease occurrence.

PCV-3 PCR positive frequency was significantly lower in feces than in tissue samples; therefore, feces were apparently not a suitable indicator of the level of PCV-3 infection. However, the presence of the virus in feces allows speculating on some routes of viral shedding. Horizontal transmission through direct contact might be a possible route, since the virus has been found in feces (*Chapter 5*), nasal swabs and oral fluids (Kwon et al., 2017) and also in trucks transporting pigs (Franzo et al., 2018a). Vertical shedding represents another possible route of transmission; the virus has been detected in fetuses and stillborn from farms with history of reproductive failure (Faccini et al., 2017; Ku et al., 2017; Palinski et al., 2017), as well as in semen and colostrum (Kedkovid et al., 2018a). Definitively, more studies are needed to ascertain the potential excretion routes of this virus. In contrast, this information is well-known for another circovirus like PCV-2 (Grau-Roma et al., 2009; Madson and Opriessnig, 2011; Segalés et al., 2005b).

Genetic characterization was done in all studies of this Thesis through Sanger sequencing. PCV-3 genomes sequenced were found in different clusters when compared with available sequences from GenBank through phylogenetic analysis. However, the nucleotide identity among these sequences and those already existing in the GenBank was really high, indicated by the phylogenetic distance (>96% accounting for all sequences, where most of them showed >98% nucleotide identity). In consequence, and considering the analyses done with PCV-3 sequences of this Thesis were from a 22-year period, it seems that PCV-3 has remained fairly stable over the years without

an independent molecular evolution according to specific areas of the world. In fact, as an internal exploratory approach, ORF1 (659 nt) and ORF2 (337nt) from all partial sequences obtained in this Thesis were analyzed by pairwise distance showing that similarity among the genome was higher than 99% (overall average was 0.007 for ORF1 and 0.021 to ORF2) (Figures 6-1 and 6-2). The findings regarding the high similarity among PCV-3 sequences detected worldwide and in the present Thesis suggest that this virus does not really follow such high mutation rate. By definition, if such mutation rate would be high, it would have generated a higher variation of the genome, which should have been detected at least in the retrospective study (*Chapter 3*). Further studies on the evolution on PCV-3 are vital to solve out these controversies.

The first metagenomics sequence available from PCV-3 revealed low identity with *cap* and *rep* genes of PCV-1 and PCV-2 and a closer identity with other Circoviruses such as *Canine circovirus* (Palinski et al., 2017; Phan et al., 2016) and *Barbel circovirus* (Zheng et al., 2017). The *Circovirus* genus members are able to infect a wide range of hosts, and cross-species transmission has also been reported (Li et al., 2010). Franzo and collaborators (2018e) speculated the possibility of PCV-3 being the product of recombination related with a host jump. The analysis of genome composition of PCV-3 found the *rep* gene closely related with that of bat circoviruses and *cap* gene with that of avian ones (Franzo et al., 2018e). On the other hand, two separate groups (proposed as genotypes) of PCV-3, named as PCV-3a and PCV-3b, were described by Fux and collaborators (2018). They found nucleotide changes which resulted in two amino acid alterations in ORF1/ORF2 and ORF3 (A24V and R27K). Li and colleagues (2018) also suggested two groups with two individual subclades termed PCV-3a-1 and PCV-3a-2. The amino acid site 24 from ORF2, predicted to be under positive

selection, was suggested as a potential epitope. Moreover, the presence of possible genotypes was also suggested in other studies (Franzo et al., 2018b; Fu et al., 2017). However, considering the high similarity found in partial or complete PCV-3 sequences (> 98% in most of the cases), the importance of determining genotypes or groupings at this stage seems poorly relevant. Worth to mention is the fact that all sequences obtained throughout this Thesis, and the up-to-date available reports, were obtained through Sanger technology. Due to the sensitivity limitations of this technique, it must be emphasized the need to apply NGS technology to discover minor variants, which might unravel the presence of quasispecies undetected by the technology used.

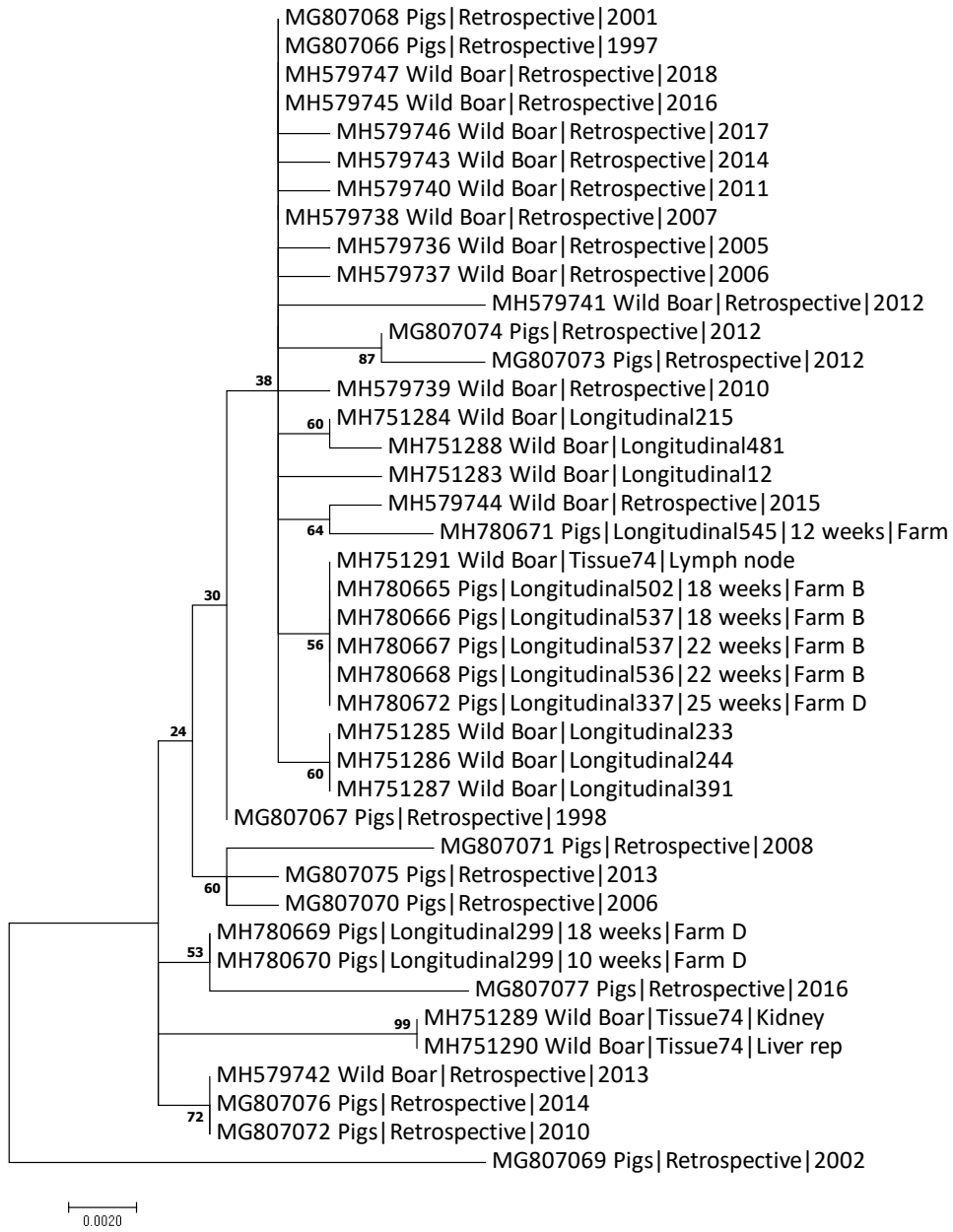

Figure 6-1. Phylogenetic tree of PCV-3 sequences based on the partial rep genomes obtained through all studies of this Thesis. The phylogenetic tree was constructed using the Maximum-likelihood algorithm of MEGA 7 Software with 1,000 bootstraps replicates.

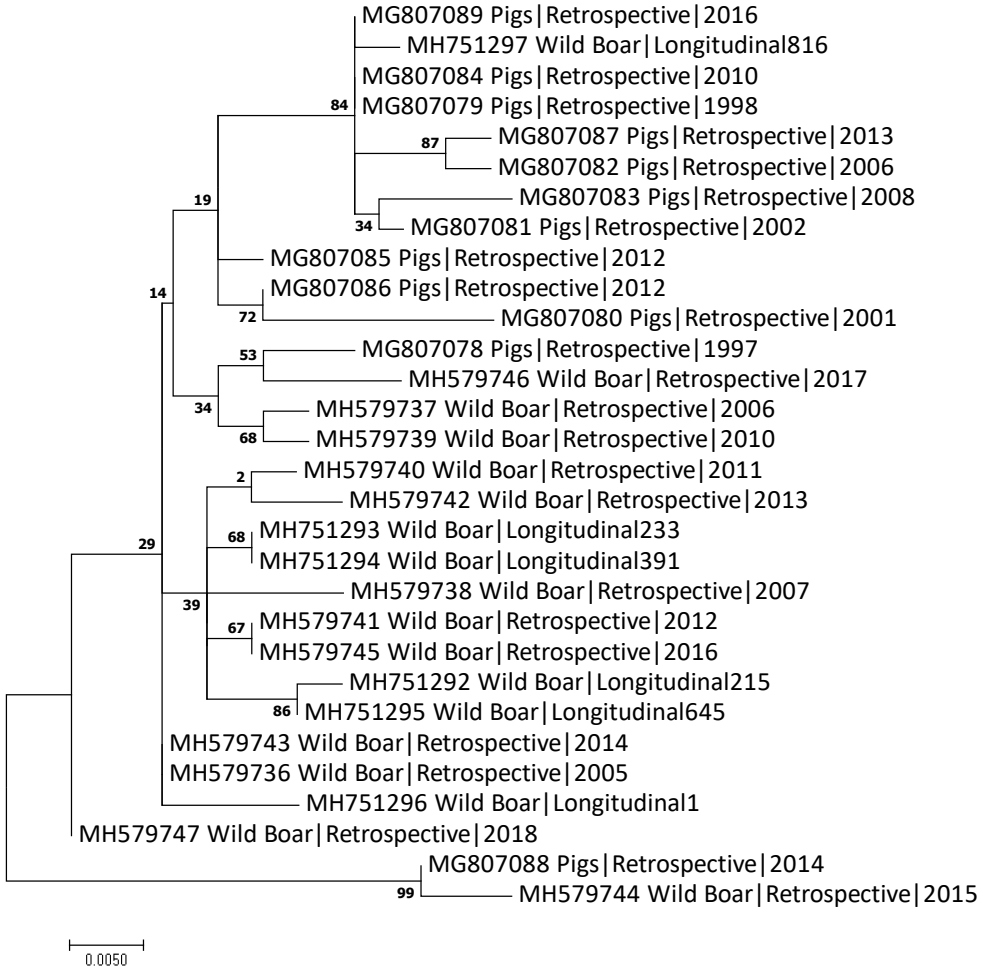

Figure 6-2. Phylogenetic tree of PCV-3 sequences based on the partial cap genomes obtained through all studies of this Thesis. The phylogenetic tree was constructed using the Maximum-likelihood algorithm of MEGA 7 Software with 1,000 bootstraps replicates.

CHAPTER 7

CONCLUSIONS

1. PCV-3, a recently discovered virus, is present in domestic pig and wild boar populations in Spain. The virus has been circulating in pig and wild boar at least since 1996 and 2004, respectively.

2. PCV-3 was not associated with any clinical and/or pathological disorders in domestic pigs or to any production phase, and was able to cause subclinical infection in apparently healthy animals. In all cases, PCV-3 genome load was low to moderate in serum samples, further indicating the subclinical nature of the infection.

3. PCV-3 circulated in all age-groups of longitudinally-monitored pigs from four different conventional farms. The highest percentage of infected animals at a given age varied among farms, with evidence of infection of more than 70% of the pigs across samplings. No specific infection dynamics pattern was determined.

4. The wild boar is susceptible to PCV-3 infection to a higher frequency than domestic pig and is able to develop a persistent infection; a potential reservoir role of the wild boar for this virus must be considered.

5. PCV-3 was detected in all tested tissue types as well as in feces of wild boar. The most frequent tissues harboring PCV-3 DNA were tonsil, lung, liver and spleen, although the highest viral load was found in submandibular lymph node. The viral load measured by qPCR was low to moderate, suggesting that wild boar were subclinically infected.

6. All PCV-3 total or partial sequences found in domestic pig and wild boar showed high similarity at nucleotide level (>98%); PCV-3 genome appears to be stable since its sequences have shown minimal variability across the analyzed years.

CHAPTER 8

REFERENCES

Afghah, Z., Webb, B., Meng, X.-J., Ramamoorthy, S., 2017. Ten years of PCV2 vaccines and vaccination: Is eradication a possibility? Vet. Microbiol. 206, 21–28. https://doi.org/https://doi.org/10.1016/j.vetmic.2016.10.002

Allan, G., Krakowka, S., Ellis, J., Charreyre, C., 2012. Discovery and evolving history of two genetically related but phenotypically different viruses, porcine circoviruses 1 and 2. Virus Res. 164, 4–9. https://doi.org/https://doi.org/10.1016/j.virusres.2011.09.013

Allan, G.M., Ellis, J.A., 2000. Porcine Circoviruses: A Review. J. Vet. Diagnostic Investig. 12, 3–14. https://doi.org/10.1177/104063870001200102

Allan, G.M., Mackie, D.P., McNair, J., Adair, B.M., McNulty, M.S., 1994a. Production, preliminary characterisation and applications of monoclonal antibodies to porcine circovirus. Vet. Immunol. Immunopathol. 43, 357–371. https://doi.org/10.1016/0165-2427(94)90157-0

Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A.C., Adair, B., Ellis, W.A., McNulty, M.S., 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. 44, 49–64. https://doi.org/10.1016/0378-1135(94)00136-K

Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of Porcine Circovirus-like Viruses from Pigs with a Wasting Disease in the USA and Europe. J. Vet. Diagnostic Investig. 10, 3–10. https://doi.org/10.1177/104063879801000102

Allan, G.M., McNeilly, F., McNair, I., Curran, M.D., Walker, I., Ellis, J., Konoby, C., Kennedy, S., Meehan, B., 2000. Absence of evidence for porcine circovirustype 2 in cattle and humans, and lack of seroconversion

or lesions in experimentally infected sheep. Arch. Virol. 145, 853–857. https://doi.org/10.1007/s007050050678

Allan, G.M., Phenix, K. V, Todd, D., McNulty, M.S., 1994b. Some Biological and Physico-Chemical Properties of Porcine Circovirus. J. Vet. Med. Ser. B 41, 17–26. https://doi.org/10.1111/j.1439-0450.1994.tb00201.x

Avert- Retrieved from https://avert.org/global-hiv-and-aids-statistics

Bao, F., Mi, S., Luo, Q., Guo, H., Tu, C., Zhu, G., Gong, W., 2017. Retrospective study of porcine circovirus type 2 infection reveals a novel genotype PCV2f. Transbound. Emerg. Dis. 65, 432–440. https://doi.org/10.1111/tbed.12721

Baró, J., Segalés, J., Martínez, J., 2015. Porcine circovirus type 2 (PCV2) enteric disease: An independent condition or part of the systemic disease? Vet. Microbiol. 176, 83–87. https://doi.org/10.1016/j.vetmic.2015.01.006

Beach, N.M., Juhan, N.M., Cordoba, L., Meng, X.J., 2010. Replacement of the Replication Factors of Porcine Circovirus (PCV) Type 2 with Those of PCV Type 1 Greatly Enhances Viral Replication In Vitro . J. Virol. 84, 8986–8989. https://doi.org/10.1128/JVI.00522-10

Bédarida, S., Dutour, O., Buzhilova, A.P., de Micco, P., Biagini, P., 2011. Identification of viral DNA (Anelloviridae) in a 200-year-old dental pulp sample (Napoleon's Great Army, Kaliningrad, 1812). Infect. Genet. Evol. 11, 358–362. https://doi.org/10.1016/j.meegid.2010.11.007

Benedictow, O.J., 2006. Ole J Benedictow, The Black Death 1346–1353: the complete history. Med. Hist. 50, 412–413. https://doi.org/10.1017/S002572730001019X

Bielanski, A., Algire, J., Larochelle, R., Magar, R., 2004. Distribution of PCV-2 DNA in the reproductive tract, oocytes and embryos of PCV-2 antibody-positive pigs. Vet. Rec. 155, 597 LP-598.

Blomström, A.-L., Belák, S., Fossum, C., McKillen, J., Allan, G., Wallgren, P., Berg, M., 2009. Detection of a novel porcine boca-like virus in the background of porcine circovirus type 2 induced postweaning multisystemic wasting syndrome. Virus Res. 146, 125–129. https://doi.org/10.1016/j.virusres.2009.09.006

Blunt, R., McOrist, S., McKillen, J., McNair, I., Jiang, T., Mellits, K., 2011. House fly vector for porcine circovirus 2b on commercial pig farms. Vet. Microbiol. 149, 452–455. https://doi.org/10.1016/j.vetmic.2010.11.019

Boadella, M., Ruiz-Fons, F.J., Vicente, J., Martín, M., Segalés, J., Gortazar, C., 2011. Seroprevalence Evolution of Selected Pathogens in Iberian Wild Boar. Transbound. Emerg. Dis. 59, 395–404. https://doi.org/10.1111/j.1865-1682.2011.01285.x

Brunborg, I.M., Jonassen, C.M., Moldal, T., Bratberg, B., Lium, B., Koenen, F., Schönheit, J., 2007. Association of myocarditis with high viral load of porcine circovirus type 2 in several tissues in cases of fetal death and high mortality in piglets. A case study. J Vet Diagn Investig 19. https://doi.org/10.1177/104063870701900405

Calsamiglia, M., Segalés, J., Quintana, J., Rosell, C., Domingo, M., 2002. Detection of Porcine Circovirus Types 1 and 2 in Serum and Tissue Samples of Pigs with and without Postweaning Multisystemic Wasting Syndrome. J. Clin. Microbiol. 40, 1848–1850. https://doi.org/10.1128/JCM.40.5.1848-1850.2002

Canning, P., Canon, A., Bates, J.L., Gerardy, K., Linhares, D.C.L., Piñeyro, P.E., Schwartz, K.J., Yoon, K.J., Rademacher, C.J., Holtkamp, D., Karriker, L., 2016. Neonatal Mortality, Vesicular Lesions and Lameness Associated with Senecavirus A in a U.S. Sow Farm. Transbound. Emerg. Dis. 63, 373–378.

https://doi.org/10.1111/tbed.12516

Cárdenas, J.P., Quatrini, R., Holmes, D.S., 2016. Genomic and metagenomic challenges and opportunities for bioleaching: a mini-review. Res. Microbiol. 167, 529–538. https://doi.org/10.1016/j.resmic.2016.06.007

Casas, V., Maloy, S., 2014. Genomic and Metagenomic Approaches for Predicting Pathogen Evolution. Microbiol. Spectr. 2.

Chen, G.H., Mai, K.J., Zhou, L., Wu, R.T., Tang, X.Y., Wu, J.L., He, L.L., Lan, T., Xie, Q.M., Sun, Y., Ma, J.Y., 2017. Detection and genome sequencing of porcine circovirus 3 in neonatal pigs with congenital tremors in South China. Transbound. Emerg. Dis. 64, 1650–1654. https://doi.org/10.1111/tbed.12702

Choi, J., Stevenson, G.W., Kiupel, M., Harrach, B., Anothayanontha, L., Kanitz, C.L., Mittal, S.K., 2002. Sequence analysis of old and new strains of porcine circovirus associated with congenital tremors in pigs and their comparison with strains involved with postweaning multisystemic wasting syndrome. Can. J. Vet. Res. 66, 217–224.

Christensen, P.B., Engle, R.E., Hjort, C., Homburg, K.M., Vach, W., Georgsen, J., Purcell, R.H., 2008. Time Trend of the Prevalence of Hepatitis E Antibodies among Farmers and Blood Donors: A Potential Zoonosis in Denmark. Clin. Infect. Dis. 47, 1026–1031. https://doi.org/10.1086/591970

Clark, G. E., 1997. Post-weaning multisystemic wasting syndrome. Proc. Am. Assoc. Swine. Pr. 28, 499–501.

Closa-Sebastià, F., Casas-Díaz, E., Cuenca, R., Lavín, S., Mentaberre, G., Marco, I., 2011. Antibodies to selected pathogens in wild boar (Sus scrofa) from Catalonia (NE Spain). Eur. J. Wildl. Res. 57, 977–981. https://doi.org/10.1007/s10344-010-0491-9

Collins, P.J., McKillen, J., Allan, G., 2017. Porcine circovirus type 3 in the UK. Vet. Rec. 181, 599 LP-599.

Conway, D.J., Roper, C., 2000. Micro-evolution and emergence of pathogens. Int. J. Parasitol. 30, 1423–1430. https://doi.org/10.1016/S0020-7519(00)00126-0

Cortey, M., Segalés, J., 2012. Low levels of diversity among genomes of Porcine circovirus type 1 (PCV1) points to differential adaptive selection between Porcine circoviruses. Virology 422, 161–164. /https://doi.org/10.1016/j.virol.2011.10.014

Crowther, R.A., Berriman, J.A., Curran, W.L., Allan, G.M., Todd, D., 2003. Comparison of the Structures of Three Circoviruses: Chicken Anemia Virus, Porcine Circovirus Type 2, and Beak and Feather Disease Virus. J. Virol. 77, 13036–13041. https://doi.org/10.1128/JVI.77.24.13036-13041.2003

Cságola, A., Cadar, D., Tuboly, T., 2008. Replication and transmission of porcine circovirus type 2 in mice. Acta Vet. Hung. 56, 421–427. https://doi.org/10.1556/AVet.56.2008.3.15

da Silva, F.M.F., Júnior, A.S., de Oliveira Peternelli, E.F., Viana, V.W., Neto, O.C., Fietto, J.L.R., Vargas, M.I., Nero, L.A., de Almeida, M.R., 2011. Retrospective study on Porcine circovirus-2 by nested pcr and real time pcr in archived tissues from 1978 in brazil. Brazilian J. Microbiol. 42, 1156–1160. https://doi.org/10.1590/S1517-838220110003000039

Darwich, L., Segalés, J., Mateu, E., 2004. Pathogenesis of postweaning multisystemic wasting syndrome caused by Porcine circovirus 2: an immune riddle. Arch. Virol. 149, 857–874. https://doi.org/10.1007/s00705-003-0280-9

Davies, B., Wang, X., Dvorak, C.M.T., Marthaler, D., Murtaugh, M.P., 2016. Diagnostic phylogenetics reveals a new Porcine circovirus 2

cluster. Virus Res. 217, 32–37. https://doi.org/10.1016/j.virusres.2016.02.010

Davies, P.R., 2012. One World, One Health: The Threat of Emerging Swine Diseases. A North American Perspective. Transbound. Emerg. Dis. 59, 18–26. https://doi.org/10.1111/j.1865-1682.2012.01312.x

Day, M.J., 2011. One health: the importance of companion animal vector-borne diseases. Parasit. Vectors 4, 49. https://doi.org/10.1186/1756-3305-4-49

Dayaram, A., Potter, K.A., Moline, A.B., Rosenstein, D.D., Marinov, M., Thomas, J.E., Breitbart, M., Rosario, K., Argüello-Astorga, G.R., Varsani, A., 2013. High global diversity of cycloviruses amongst dragonflies. J. Gen. Virol. 94, 1827–1840.

Dekkers, J., Rowland, R.R.R., Lunney, J.K., Plastow, G., 2017. Host genetics of response to porcine reproductive and respiratory syndrome in nursery pigs. Vet. Microbiol. 209, 107–113. https://doi.org/10.1016/j.vetmic.2017.03.026

Deng, J., Li, X., Zheng, D., Wang, Y., Chen, L., Song, H., Wang, T., Huang, Y., Pang, W., Tian, K., 2018. Establishment and application of an indirect ELISA for porcine circovirus 3. Arch. Virol. 163, 479–482. https://doi.org/10.1007/s00705-017-3607-7

Drolet, R., Thibault, S., D'Allaire, S., Thompson, J., Done, S., 1999. Porcine dermatitis and nephropathy syndrome (PDNS): an overview of the disease. Swine Health and Prod. 7(6), 283- 285.

Dulac, G.C., Afshar, A., 1989. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. 53, 431—433.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., Relman, D.A., 2005. Diversity of

the Human Intestinal Microbial Flora. Science 308, 1635–1638. https://doi.org/10.1126/science.1110591

Edfors-Lilja, I., Wattrang, E., Marklund, L., Moller, M., Andersson-Eklund, L., Andersson, L., Fossum, C., 1998. Mapping Quantitative Trait Loci for Immune Capacity in the Pig. J. Immunol. 161, 829–835.

Ellis, J., Spinato, M., Yong, C., West, K., McNeilly, F., Meehan, B., Kennedy, S., Clark, E., Krakowka, S., Allan, G., 2003. Porcine Circovirus 2–Associated Disease in Eurasian Wild Boar. J. Vet. Diagnostic Investig. 15, 364–368. https://doi.org/10.1177/104063870301500411

Ellis, J.A., Konoby, C., West, K.H., Allan, G.M., Krakowka, S., McNeilly, F., Meehan, B., Walker, I., 2001. Lack of antibodies to porcine circovirus type 2 virus in beef and dairy cattle and horses in western Canada. Can. Vet. J. 42, 461–464.

Fablet, C., Rose, N., Bernard, C., Messager, I., Piel, Y., Grasland, B., 2017. Estimation of the diagnostic performance of two ELISAs to detect PCV2 antibodies in pig sera using a Bayesian method. J. Virol. Methods 249, 121–125. https://doi.org/10.1016/j.jviromet.2017.09.002

Faccini, S., Barbieri, I., Gilioli, A., Sala, G., Gibelli, L.R., Moreno, A., Sacchi, C., Rosignoli, C., Franzini, G., Nigrelli, A., 2017. Detection and genetic characterization of Porcine circovirus type 3 in Italy. Transbound. Emerg. Dis. 64, 1661–1664. https://doi.org/10.1111/tbed.12714

Fan, S., Ku, X., Chen, F., Wang, Y., Yu, X., He, Q., 2017. Complete Genome Sequence of a Novel Porcine Circovirus Type 3 Strain, PCV3/CN/Hubei-618/2016, Isolated from China. Genome Announc. https://doi.org/10.1128/genomeA.00100-17

Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.-J., 2000a. Genetic Characterization of Type 2 Porcine Circovirus (PCV-2) from Pigs with

Postweaning Multisystemic Wasting Syndrome in Different Geographic Regions of North America and Development of a Differential PCR-Restriction Fragment Length Polymorphism Assay T. J. Clin. Microbiol. 38, 2494–2503.

Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.-J., 2000b. Genetic Characterization of Type 2 Porcine Circovirus (PCV-2) from Pigs with Postweaning Multisystemic Wasting Syndrome in Different Geographic Regions of North America and Development of a Differential PCR-Restriction Fragment Length Polymorphism Assay . J. Clin. Microbiol. 38, 2494–2503.

Fenaux, M., Halbur, P.G., Haqshenas, G., Royer, R., Thomas, P., Nawagitgul, P., Gill, M., Toth, T.E., Meng, X.J., 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J Virol 76. https://doi.org/10.1128/JVI.76.2.541-551.2002

Fenaux, M., Opriessnig, T., Halbur, P.G., Meng, X.J., 2003. Immunogenicity and Pathogenicity of Chimeric Infectious DNA Clones of Pathogenic Porcine Circovirus Type 2 (PCV2) and Nonpathogenic PCV1 in Weanling Pigs. J. Virol. 77, 11232–11243. https://doi.org/10.1128/JVI.77.20.11232-11243.2003

Feng, H., Blanco, G., Segalés, J., Sibila, M., 2014. Can Porcine circovirus type 2 (PCV2) infection be eradicated by mass vaccination? Vet. Microbiol. 172, 92–99. https://doi.org/10.1016/j.vetmic.2014.05.003

Feng, H., Segalés, J., Fraile, L., López-Soria, S., Sibila, M., 2016. Effect of high and low levels of maternally derived antibodies on porcine circovirus type 2 (PCV2) infection dynamics and production parameters in PCV2 vaccinated pigs under field conditions. Vaccine 34, 3044–3050.

https://doi.org/10.1016/j.vaccine.2016.04.088

Fernández-Aguilar, X., Gottschalk, M., Aragon, V., Càmara, J., Ardanuy, C., Velarde, R., Galofré-Milà, N., Castillo-Contreras, R., López-Olvera, J.R., Mentaberre, G., Colom-Cadena, A., Lavín, S., Cabezón, O., 2018. Urban Wild Boars and Risk for Zoonotic Streptococcus suis in Spain. Emerg. Infect. Dis. J. 24, 1083. https://doi.org/10.3201/eid2406.171271

Finsterbusch, T., Mankertz, A., 2009. Porcine circoviruses--small but powerful. Virus Res 143. https://doi.org/10.1016/j.virusres.2009.02.009

Firth, C., Charleston, M.A., Duffy, S., Shapiro, B., Holmes, E.C., 2009. Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. J. Virol. https://doi.org/10.1128/JVI.01719-09

Fort, M., Olvera, A., Sibila, M., Segalés, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Vet Microbiol. 125. https://doi.org/10.1016/j.vetmic.2007.06.004

Fournié, G., Kearsley-Fleet, L., Otte, J., Pfeiffer, D.U., 2015. Spatiotemporal trends in the discovery of new swine infectious agents. Vet. Res. 46, 114. https://doi.org/10.1186/s13567-015-0226-8

Fraile, L., Sibila, M., Nofrarías, M., López-Jimenez, R., Huerta, E., Llorens, A., López-Soria, S., Pérez, D., Segalés, J., 2012. Effect of sow and piglet porcine circovirus type 2 (PCV2) vaccination on piglet mortality, viraemia, antibody titre and production parameters. Vet. Microbiol. 161, 229–234. https://doi.org/10.1016/j.vetmic.2012.07.021

Franzo, G., Cortey, M., Castro, A.M.M.G. de, Piovezan, U., Szabo, M.P.J., Drigo, M., Segalés, J., Richtzenhain, L.J., 2015a. Genetic characterisation of Porcine circovirus type 2 (PCV2) strains from feral pigs in the Brazilian Pantanal: An opportunity to reconstruct the history

of PCV2 evolution. Vet. Microbiol. 178, 158–162. https://doi.org/10.1016/j.vetmic.2015.05.003

Franzo, G., Cortey, M., Olvera, A., Novosel, D., De Castro, A.M.M.G., Biagini, P., Segalés, J., Drigo, M., 2015b. Revisiting the taxonomical classification of Porcine Circovirus type 2 (PCV2): still a real challenge. Virol. J. 12, 131. https://doi.org/10.1186/s12985-015-0361-x

Franzo, G., Legnardi, M., Centelleghe, C., Tucciarone, C.M., Cecchinato, M., Cortey, M., Segalés, J., Drigo, M., 2018a. Development and validation of direct PCR and quantitative PCR assays for the rapid, sensitive, and economical detection of porcine circovirus 3. J. Vet. Diagnostic Investig. 1040638718770495. https://doi.org/10.1177/1040638718770495

Franzo, G., Legnardi, M., Hjulsager C., K., Klaumann, F., Larsen L., E., Segales, J., Drigo, M., 2018b. Full-genome sequencing of porcine circovirus 3 field strains from Denmark, Italy and Spain demonstrates a high within-Europe genetic heterogeneity. Transbound. Emerg. Dis. 65, 602–606. https://doi.org/10.1111/tbed.12836

Franzo, G., Legnardi, M., Tucciarone, C.M., Drigo, M., Klaumann, F., Sohrmann, M., SegalÉs, J., 2018c. Porcine circovirus type 3: a threat to the pig industry? Vet. Rec. 182, 83 LP-83.

Franzo, G., Maria, T.C., Michele, D., Mattia, C., Marco, M., Alessandra, M., Luisa, M.M., 2018d. First report of wild boar susceptibility to Porcine circovirus type 3: High prevalence in the Colli Euganei Regional Park (Italy) in the absence of clinical signs. Transbound. Emerg. Dis. 0. https://doi.org/10.1111/tbed.12905

Franzo, G., Segales, J., Tucciarone, C.M., Cecchinato, M., Drigo, M., 2018e. The analysis of genome composition and codon bias reveals distinctive patterns between avian and mammalian circoviruses which

suggest a potential recombinant origin for Porcine circovirus 3. PLoS One 13, e0199950. https://doi.org/10.1371/journal.pone.0199950

Franzo, G., Tucciarone, C.M., Dotto, G., Gigli, A., Ceglie, L., Drigo, M., 2015c. International trades, local spread and viral evolution: The case of porcine circovirus type 2 (PCV2) strains heterogeneity in Italy. Infect. Genet. Evol. https://doi.org/10.1016/j.meegid.2015.04.004

Fu, X., Fang, B., Ma, J., Liu, Y., Bu, D., Zhou, P., Wang, H., Jia, K., Zhang, G., 2017. Insights into the epidemic characteristics and evolutionary history of the novel porcine circovirus type 3 in southern China. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12752

Fux, R., Söckler, C., Link, E.K., Renken, C., Krejci, R., Sutter, G., Ritzmann, M., Eddicks, M., 2018. Full genome characterization of porcine circovirus type 3 isolates reveals the existence of two distinct groups of virus strains. Virol. J. 15, 25. https://doi.org/10.1186/s12985-018-0929-3

Gao, Z., Dong, Q., Jiang, Y., Opriessnig, T., Wang, J., Quan, Y., Yang, Z., 2014. ORF4-protein deficient PCV2 mutants enhance virus-induced apoptosis and show differential expression of mRNAs in vitro. Virus Res. 183, 56–62. https://doi.org/https://doi.org/10.1016/j.virusres.2014.01.024

Ge, X., Li, J., Peng, C., Wu, L., Yang, X., Wu, Y., Zhang, Y., Shi, Z., 2011. Genetic diversity of novel circular ssDNA viruses in bats in China. J. Gen. Virol. 92, 2646–2653.

Gibbs, E.P.J., 2014. The evolution of One Health: a decade of progress and challenges for the future. Vet. Rec. 174, 85–91. https://doi.org/10.1136/vr.g143

Gillespie, J., Opriessnig, T., Meng, X.J., Pelzer, K., Buechner-Maxwell, V., 2009. Porcine Circovirus Type 2 and Porcine Circovirus-Associated Disease. J. Vet. Intern. Med. 23, 1151–1163.

https://doi.org/10.1111/j.1939-1676.2009.0389.x

Grau-Roma, L., Crisci, E., Sibila, M., López-Soria, S., Nofrarias, M., Cortey, M., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet Microbiol. 128. https://doi.org/10.1016/j.vetmic.2007.09.007

Grau-Roma, L., Fraile, L., Segalés, J., 2011. Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. Vet. J. 187, 23–32. https://doi.org/10.1016/j.tvjl.2010.01.018

Grau-Roma, L., Hjulsager, C.K., Sibila, M., Kristensen, C.S., López-Soria, S., Enøe, C., 2009. Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark. Vet Microbiol. 135. https://doi.org/10.1016/j.vetmic.2008.10.007

Grierson, S.S., King, D.P., Wellenberg, G.J., Banks, M., 2004. Genome sequence analysis of 10 Dutch porcine circovirus type 2 (PCV-2) isolates from a PMWS case-control study. Res. Vet. Sci. 77, 265–268. https://doi.org/10.1016/j.rvsc.2004.04.008

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Guo, L., Lu, Y., Wei, Y., Huang, L., Liu, C., 2010. Porcine circovirus type 2 (PCV2): genetic variation and newly emerging genotypes in China. Virol J. 7. https://doi.org/10.1186/1743-422X-7-273

Ha, Y., Ahn, K.K., Kim, B., Cho, K.-D., Lee, B.H., Oh, Y.-S., Kim, S.-H.,

Chae, C., 2009. Evidence of shedding of porcine circovirus type 2 in milk from experimentally infected sows. Res. Vet. Sci. 86, 108–110. https://doi.org/10.1016/j.rvsc.2008.04.004

Hamel, A.L., Lin, L.L., Nayar, G.S.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol.

Han, S., Xiao, Y., Zheng, D., Gu, Y., Xuan, Y., Jin, Y., Pang, W., Huang, Y., Li, X., Deng, J., Tian, K., 2016. Establishment and application of a competitive enzyme-linked immunosorbent assay differentiating PCV2 antibodies from mixture of PCV1/PCV2 antibodies in pig sera. BMC Vet. Res. 12, 175. https://doi.org/10.1186/s12917-016-0802-9

Harding J.C.S., 1996. Postweaning multisystemic wasting syndrome (PMWS), preliminary epidemiology and clinical findings. Proc of the West Canadian Association of Swine Practitioners, p. 21

H**arding, J.C.S., Baker, C.D., Tumber, A., McIntosh, K.A., Parker, S.E., Middleton, D.M., Hill, J.E., Ellis, J.A., Krakowka, S.,** 2008. Porcine Circovirus-2 DNA Concentration Distinguishes Wasting from Nonwasting Pigs and is Correlated with Lesion Distribution, Severity, and Nucleocapsid Staining Intensity. J. Vet. Diagnostic Investig. 20, 274–282. https://doi.org/10.1177/104063870802000303

Hassing, A.-G., Wachman, H., Bækbo, P., Bøtner, A., Nielsen, J., Vigre, H., Feenstra, A., 2006. Effect of PMWS pig serum and PCV2 specific serum on mortality and weigth gain in PMWS affected herds. Proceeding 19th Int. Pig Vet. Congr.

Hause, B.M., Collin, E.A., Peddireddi, L., Yuan, F., Chen, Z., Hesse, R.A., Gauger, P.C., Clement, T., Fang, Y., Anderson, G., 2015. Discovery of a novel putative atypical porcine pestivirus in pigs in the USA. J. Gen. Virol. 96, 2994–2998.

He, J., Cao, J., Zhou, N., Jin, Y., Wu, J., Zhou, J., 2013. Identification and Functional Analysis of the Novel ORF4 Protein Encoded by Porcine Circovirus Type 2. J. Virol. https://doi.org/10.1128/JVI.01443-12

Hjulsager, C.K., Grau-Roma, L., Sibila, M., Enøe, C., Larsen, L., Segalés, J., 2009. Inter-laboratory and inter-assay comparison on two real-time PCR techniques for quantification of PCV2 nucleic acid extracted from field samples. Vet. Microbiol. 133, 172–178. https://doi.org/https://doi.org/10.1016/j.vetmic.2008.06.014

Holmes, E.C., Rambaut, A., 2004. Viral evolution and the emergence of SARS coronavirus. Philos. Trans. R. Soc. B Biol. Sci. 359, 1059–1065. https://doi.org/10.1098/rstb.2004.1478

Hsiung, G.D., 1984. Diagnostic virology: from animals to automation. Yale J. Biol. Med. 57, 727–733.

Huang, C., Hung, J.-J., Wu, C.-Y., Chien, M.-S., 2004. Multiplex PCR for rapid detection of pseudorabies virus, porcine parvovirus and porcine circoviruses. Vet. Microbiol. 101, 209–214. https://doi.org/10.1016/j.vetmic.2004.04.007

Huang, L.P., Lu, Y.H., Wei, Y.W., Guo, L.J., Liu, C.M., 2011. Identification of one critical amino acid that determines a conformational neutralizing epitope in the capsid protein of porcine circovirus type 2. BMC Microbiol. 11. https://doi.org/10.1186/1471-2180-11-188

Huang, Y., Shao, M., Xu, X., Zhang, X., Du, Q., Zhao, X., 2013. Evidence for different patterns of natural inter-genotype recombination between two PCV2 parental strains in the field. Virus Res. 175. https://doi.org/10.1016/j.virusres.2013.03.014

International Comitee on Taxonomy of Viruses- ICVT. Retrieved from https.//www.ncbi.nlm.nih.gov/ICTVdb/

Jacobsen, B., Krueger, L., Seeliger, F., Bruegmann, M., Segalés, J.,

Baumgaertner, W., 2009. Retrospective study on the occurrence of porcine circovirus 2 infection and associated entities in Northern Germany. Vet. Microbiol. 138, 27–33. https://doi.org/10.1016/j.vetmic.2009.02.005

Kappe, E.C., Halami, M.Y., Schade, B., Alex, M., Hoffmann, D., Gangl, A., Meyer, K., Dekant, W., Schwarz, B.A., Johne, R., Buitkamp, J., Böttcher, J., Müller, H., 2010. Bone marrow depletion with haemorrhagic diathesis in calves in Germany: characterization of the disease and preliminary investigations on its aetiology. Berl. Munch. Tierarztl. Wochenschr. 123, 31–41.

Karesh, W.B., Dobson, A., Lloyd-Smith, J.O., Lubroth, J., Dixon, M.A., Bennett, M., Aldrich, S., Harrington, T., Formenty, P., Loh, E.H., Machalaba, C.C., Thomas, M.J., Heymann, D.L., 2012. Ecology of zoonoses: natural and unnatural histories. Lancet 380, 1936–1945. https://doi.org/10.1016/S0140-6736(12)61678-X

Karuppannan, A.K., Kwang, J., 2011. ORF3 of porcine circovirus 2 enhances the in vitro and in vivo spread of the of the virus. Virology 410, 248–256. https://doi.org/https://doi.org/10.1016/j.virol.2010.11.009

Karuppannan, A.K., Opriessnig, T., 2017. Porcine Circovirus Type 2 (PCV2) Vaccines in the Context of Current Molecular Epidemiology. Viruses 9, 99. https://doi.org/10.3390/v9050099

Kedkovid, R., Woonwong, Y., Arunorat, J., Sirisereewan, C., Sangpratum, N., Kesdangsakonwut, S., Tummaruk, P., Teankum, K., Assavacheep, P., Jittimanee, S., Thanawongnuwech, R., 2018a. Porcine circovirus type 3 (PCV3) shedding in sow colostrum. Vet. Microbiol. 220, 12–17. https://doi.org/10.1016/j.vetmic.2018.04.032

Kedkovid, R., Woonwong, Y., Arunorat, J., Sirisereewan, C., Sangpratum, N., Lumyai, M., Kesdangsakonwut, S., Teankum, K.,

Jittimanee, S., Thanawongnuwech, R., 2018b. Porcine circovirus type 3 (PCV3) infection in grower pigs from a Thai farm suffering from porcine respiratory disease complex (PRDC). Vet. Microbiol. 215, 71–76. https://doi.org/10.1016/j.vetmic.2018.01.004

Kekarainen, T., Segalés, J., 2015. Porcine circovirus 2 immunology and viral evolution. Porc. Heal. Manag. https://doi.org/10.1186/s40813-015-0012-z

Kim, H.-R., Park, Y.-R., Lim, D.-R., Park, M.-J., Park, J.-Y., Kim, S.-H., Lee, K.-K., Lyoo, Y.S., Park, C.-K., 2017. Multiplex real-time polymerase chain reaction for the differential detection of porcine circovirus 2 and 3. J. Virol. Methods 250, 11–16. https://doi.org/10.1016/j.jviromet.2017.09.021

Kiupel, M., Stevenson, G.W., Choi, J., Latimer, K.S., Kanitz, C.L., Mittal, S.K., 2001. Viral Replication and Lesions in BALB/c Mice Experimentally Inoculated with Porcine Circovirus Isolated from a Pig with Postweaning Multisystemic Wasting Disease. Vet. Pathol. 38, 74–82. https://doi.org/10.1354/vp.38-1-74

Knell, S., Willems, H., Hertrampf, B., Reiner, G., 2005. Comparative genetic characterization of Porcine Circovirus type 2 samples from German wild boar populations. Vet. Microbiol. 109, 169–177. https://doi.org/10.1016/j.vetmic.2005.06.004

Krakowka, S., Ellis, J.A., Meehan, B., Kennedy, S., McNeilly, F., Allan, G., 2000. Viral Wasting Syndrome of Swine: Experimental Reproduction of Postweaning Multisystemic Wasting Syndrome in Gnotobiotic Swine by Coinfection with Porcine Circovirus 2 and Porcine Parvovirus. Vet. Pathol. 37, 254–263. https://doi.org/10.1354/vp.37-3-254

Ku, X., Chen, F., Li, P., Wang, Y., Yu, X., Fan, S., Qian, P., Wu, M., He, Q., 2017. Identification and genetic characterization of porcine circovirus

type 3 in China. Transbound. Emerg. Dis. 64, 703–708. https://doi.org/10.1111/tbed.12638

Kumar, D., Beach, N.M., Meng, X.-J., Hegde, N.R., 2012. Use of PCR-based assays for the detection of the adventitious agent porcine circovirus type 1 (PCV1) in vaccines, and for confirming the identity of cell substrates and viruses used in vaccine production. J. Virol. Methods 179, 201–211. https://doi.org/10.1016/j.jviromet.2011.10.017

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874.

Kwon, T., Yoo, S.J., Park, C.-K., Lyoo, Y.S., 2017. Prevalence of novel porcine circovirus 3 in Korean pig populations. Vet. Microbiol. 207, 178–180. https://doi.org/10.1016/j.vetmic.2017.06.013

La Rosa, G., Fratini, M., della Libera, S., Iaconelli, M., Muscillo, M., 2012. Emerging and potentially emerging viruses in water environments. Ann Ist Super Sanità 48, 397–406. https://doi.org/10.4415/ANN_12_04_07

Labarque, G.G., Nauwynck, H.J., Mesu, A.P., Pensaert, M.B., 2000. Seroprevalence of porcine circovirus types 1 and 2 in the Belgian pig population. Vet. Q. 22, 234–236. https://doi.org/10.1080/01652176.2000.9695065

Lan, D., Ji, W., Yang, S., Cui, L., Yang, Z., Yuan, C., Hua, X., 2011. Isolation and characterization of the first Chinese porcine sapelovirus strain. Arch. Virol. 156, 1567. https://doi.org/10.1007/s00705-011-1035-7

Larochelle, R., Bielanski, A., Müller, P., Magar, R., 2000. PCR Detection and Evidence of Shedding of Porcine Circovirus Type 2 in Boar Semen. J. Clin. Microbiol. 38, 4629–4632.

Larochelle, R., Magar, R., D'Allaire, S., 2003. Comparative serologic and

virologic study of commercial swine herds with and without postweaning multisystemic wasting syndrome. Can J Vet Res. 67.

LeCann, P., Albina, E., Madec, F., Cariolet, R., Jestin, A., 1997. Piglet wasting disease. Veterinary Record 141: 660

Lefebvre, D.J., Van Doorsselaere, J., Delputte, P.L., Nauwynck, H.J., 2009. Recombination of two porcine circovirus type 2 strains. Arch. Virol. 154, 875–879. https://doi.org/10.1007/s00705-009-0379-8

Lepage, P., Leclerc, M.C., Joossens, M., Mondot, S., Blottière, H.M., Raes, J., Ehrlich, D., Doré, J., 2013. A metagenomic insight into our gut{\textquoteright}s microbiome. Gut 62, 146–158. https://doi.org/10.1136/gutjnl-2011-301805

Lerner, H., Berg, C., 2015. The concept of health in One Health and some practical implications for research and education: what is One Health? Infect. Ecol. Epidemiol. 5, 10.3402/iee.v5.25300. https://doi.org/10.3402/iee.v5.25300

Li, L., Kapoor, A., Slikas, B., Bamidele, O.S., Wang, C., Shaukat, S., Masroor, M.A., Wilson, M.L., Ndjango, J.-B.N., Peeters, M., Gross-Camp, N.D., Muller, M.N., Hahn, B.H., Wolfe, N.D., Triki, H., Bartkus, J., Zaidi, S.Z., Delwart, E., 2010. Multiple Diverse Circoviruses Infect Farm Animals and Are Commonly Found in Human and Chimpanzee Feces. J. Virol. https://doi.org/10.1128/JVI.02109-09

Li, L., McGraw, S., Zhu, K., Leutenegger, C.M., Marks, S.L., Kubiski, S., Gaffney, P., Dela Cruz Jr, F.N., Wang, C., Delwart, E., Pesavento, P.A., 2013. Circovirus in Tissues of Dogs with Vasculitis and Hemorrhage. Emerg. Infect. Dis. 19, 534–541. https://doi.org/10.3201/eid1904.121390

Li, L., Victoria, J.G., Wang, C., Jones, M., Fellers, G.M., Kunz, T.H., Delwart, E., 2010. Bat Guano Virome: Predominance of Dietary Viruses

from Insects and Plants plus Novel Mammalian Viruses . J. Virol. 84, 6955–6965. https://doi.org/10.1128/JVI.00501-10

Li, X., Bai, Y., Zhang, H., Zheng, D., Wang, T., Wang, Y., Deng, J., Sun, Z., Tian, K., 2018a. Production of a monoclonal antibody against Porcine circovirus type 3 cap protein. J. Virol. Methods 261, 10–13. https://doi.org/10.1016/j.jviromet.2018.07.014

Li, X., Qiao, M., Sun, M., Tian, K., 2018b. A Duplex Real-Time PCR Assay for the Simultaneous Detection of Porcine Circovirus 2 and Circovirus 3. Virol. Sin. 33, 181–186. https://doi.org/10.1007/s12250-018-0025-2

Lian, H., Liu, Y., Li, N., Wang, Y., Zhang, S., Hu, R., 2014. Novel Circovirus from Mink, China. Emerg. Infect. Dis. 20, 1548–1550. https://doi.org/10.3201/eid2009.140015

Liang, H., Su, S., Deng, S., Gu, H., Ji, F., Wang, L., Liang, C., Wang, H., Zhang, G., 2014. The Prevalence of Hepatitis E Virus Infections among Swine, Swine Farmers and the General Population in Guangdong Province, China. PLoS One 9, e88106. https://doi.org/10.1371/journal.pone.0088106

Lima, F.E.S., Cibulski, S.P., Dall Bello, A.G., Mayer, F.Q., Witt, A.A., Roehe, P.M., Azevedo, P.A., 2015. A Novel Chiropteran Circovirus Genome Recovered from a Brazilian Insectivorous Bat Species. Microbiol. Resour. Announc. 3. https://doi.org/10.1128/genomeA.01393-15

Lin, C., Gu, J., Wang, H., Zhou, J., Li, J., Wang, S., Jin, Y., Liu, C., Liu, J., Yang, H., Jiang, P., Zhou, J., 2018. Caspase-Dependent Apoptosis Induction via Viral Protein ORF4 of Porcine Circovirus 2 Binding to Mitochondrial Adenine Nucleotide Translocase 3. J. Virol. 92. https://doi.org/10.1128/JVI.00238-18

Lin, W.-L., Chien, M.-S., Wu, P.-C., Lai, C.-L., Huang, C., 2011. The Porcine

Circovirus Type 2 Nonstructural Protein ORF3 Induces Apoptosis in Porcine Peripheral Blood Mononuclear Cells. Open Virol. J. 5, 148–153. https://doi.org/10.2174/1874357901105010148

Lipej, Z., Segalés, J., Jemeršić, L., Olvera, A., Roić, B., Novosel, D., Mihaljević, Ž., Manojlović, L., 2007. First description of postweaning multisystemic wasting syndrome (PMWS) in wild boar (Sus scrofa) in Croatia and phylogenetic analysis of partial PCV2 sequences. Acta Vet. Hung. 55, 389–404. https://doi.org/10.1556/AVet.55.2007.3.13

Liu, J., Chen, I., Kwang, J., 2005. Characterization of a Previously Unidentified Viral Protein in Porcine Circovirus Type 2-Infected Cells and Its Role in Virus-Induced Apoptosis. J. Virol. 79, 8262–8274. https://doi.org/10.1128/JVI.79.13.8262-8274.2005

Lőrincz, M., Cságola, A., Biksi, I., Szeredi, L., Dán, Á., Tuboly, T., 2010. Detection of porcine circovirus in rodents — Short communication. Acta Vet. Hung. 58, 265–268. https://doi.org/10.1556/AVet.58.2010.2.12

Lőrincz, M., Dán, Á., Láng, M., Csaba, G., Tóth, Á.G., Székely, C., Cságola, A., Tuboly, T., 2012. Novel circovirus in European catfish (Silurus glanis). Arch. Virol. 157, 1173–1176. https://doi.org/10.1007/s00705-012-1291-1

Lukert, P., de Boer, G. F., Dale, J. L., Keese, P., McNulty, M. S., Randles, J. W., Tischer, I., 1995. The *Circoviridae*. In *Virus Taxonomy*. *Sixth Report of the International Committee on Taxonomy of Viruses*, pp. 166-168. Edited by F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo & M. D. Summers. Vienna & New York: Springer-Verlag

Madec, F., Rose, N., Grasland, B., Cariolet, R., Jestin, A., 2008. Post-Weaning Multisystemic Wasting Syndrome and Other PCV2-Related Problems in Pigs: a 12-Year Experience. Transbound. Emerg. Dis. 55,

273–283. https://doi.org/10.1111/j.1865-1682.2008.01035.x

Madson, D.M., Opriessnig, T., 2011. Effect of porcine circovirus type 2 (PCV2) infection on reproduction: disease, vertical transmission, diagnostics and vaccination. Anim. Heal. Res. Rev. 12, 47–65. https://doi.org/10.1017/S1466252311000053

Mandrioli, L., Sarli, G., Panarese, S., Baldoni, S., Marcato, P.S., 2004. Apoptosis and proliferative activity in lymph node reaction in postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. 97, 25–37. https://doi.org/https://doi.org/10.1016/j.vetimm.2003.08.017

Mankertz, A., Çaliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T., Finsterbusch, T., 2004. Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. Vet. Microbiol. 98, 81–88. https://doi.org/10.1016/j.vetmic.2003.10.014

Mankertz, A., Domingo, M., Folch, J.M., LeCann, P., Jestin, A., Segalés, J., Chmielewicz, B., Plana-Durán, J., Soike, D., 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. 66, 65–77. https://doi.org/10.1016/S0168-1702(99)00122-7

Mankertz, A., Mankertz, J., Wolf, K., Buhk, H.J., 1998. Identification of a protein essential for replication of porcine circovirus. J. Gen. Virol. https://doi.org/10.1099/0022-1317-79-2-381

Mankertz, A., Persson, F., Mankertz, J., Blaess, G., Buhk, H.J., 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. J. Virol. 71, 2562–2566.

Mason, O.U., Scott, N.M., Gonzalez, A., Robbins-Pianka, A., Bælum, J., Kimbrel, J., Bouskill, N.J., Prestat, E., Borglin, S., Joyner, D.C., Fortney, J.L., Jurelevicius, D., Stringfellow, W.T., Alvarez-Cohen,

L., Hazen, T.C., Knight, R., Gilbert, J.A., Jansson, J.K., 2014. Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. ISME J. 8, 1464–1475. https://doi.org/10.1038/ismej.2013.254

Mateusen, B., Maes, D.G.D., Van Soom, A., Lefebvre, D., Nauwynck, H.J., 2007. Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. Theriogenology 68, 896–901. https://doi.org/10.1016/j.theriogenology.2007.07.014

McNair, I., McNelly, F., Duffy, C., Tregaskis, P., McKay, P., Fossum, C., Ellis, J., Krakowka, S., Allan, G.M., 2007. Early distribution of PCV2 in a PMWS disease model. In: Proceddings of the 5th International Symposium on Emerging and Reemerging Pig Diseases, Krakow, Poland, p.56

McIntosh, K.A., Harding, J.C.S., Ellis, J.A., Appleyard, G.D., 2006. Detection of Porcine circovirus type 2 viremia and seroconversion in naturally infected pigs in a farrow-to-finish barn. Can. J. Vet. Res. 70, 58–61.

Meehan, B.M., Creelan, J.L., McNulty, M.S., Todd, D., 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. 78, 221–227.

Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. 79, 2171–2179.

Meerts, P., Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H., 2005. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. Viral Immunol. 18. https://doi.org/10.1089/vim.2005.18.333

Meng, X.J., 2012. Emerging and Re-emerging Swine Viruses. Transbound. Emerg. Dis. 59, 85–102. https://doi.org/10.1111/j.1865-1682.2011.01291.x

Meng, X.J., Lindsay, D.S., Sriranganathan, N., 2009. Wild boars as sources for infectious diseases in livestock and humans. Philos. Trans. R. Soc. B Biol. Sci. 364, 2697–2707. https://doi.org/10.1098/rstb.2009.0086

Meslin, F.-X., Stohr, K. & Heymann, D. 2000 Public health implications of emerging zoonoses. Rev. Sci. Tech. d'OIE 19, 310-317.

Morens, D.M., Fauci, A.S., 2013. Emerging Infectious Diseases: Threats to Human Health and Global Stability. PLoS Pathog. 9, e1003467. https://doi.org/10.1371/journal.ppat.1003467

Morens, D.M., Folkers, G.K., Fauci, A.S., 2004. The challenge of emerging and re-emerging infectious diseases. Nature 430, 242.

Morse, S.S., 2012. Public Health Surveillance and Infectious Disease Detection. Biosecurity Bioterrorism Biodefense Strateg. Pract. Sci. 10, 6–16. https://doi.org/10.1089/bsp.2011.0088

Morse, S.S., Mazet, J.A.K., Woolhouse, M., Parrish, C.R., Carroll, D., Karesh, W.B., Zambrana-Torrelio, C., Lipkin, W.I., Daszak, P., 2012. Prediction and prevention of the next pandemic zoonosis. Lancet 380, 1956–1965. https://doi.org/10.1016/S0140-6736(12)61684-5

Muhling, J., Raye, W.S., Buddle, J.R., Wilcox, G.E., 2006. Genetic characterisation of Australian strains of porcine circovirus types 1 and 2. Aust. Vet. J. 84, 421–425. https://doi.org/10.1111/j.1751-0813.2006.00081.x

Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., Paul, P.S., 2002. Modified Indirect Porcine Circovirus (PCV) Type 2-Based and Recombinant Capsid Protein (ORF2)-Based Enzyme-Linked Immunosorbent Assays for Detection of

Antibodies to PCV. Clin. Diagn. Lab. Immunol. 9, 33–40. https://doi.org/10.1128/CDLI.9.1.33-40.2002

Nayar, G.P., Hamel, A.L., Lin, L., Sachvie, C., Grudeski, E., Spearman, G., 1999. Evidence for circovirus in cattle with respiratory disease and from aborted bovine fetuses. Can. Vet. J. 40, 277–278.

Nichol, S.T., Arikawa, J., Kawaoka, Y., 2000. Emerging viral diseases. Proc. Natl. Acad. Sci. U. S. A. 97, 12411–12412.

Nielsen, E.O., Enøe, C., Jorsal, S.E., Barfod, K., Svensmark, B., Bille-Hansen, V., Vigre, H., Bøtner, A., Baekbo, P., 2008. Postweaning multisystemic wasting syndrome in Danish pig herds: productivity, clinical signs and pathology. Vet. Rec. 162, 505–508. https://doi.org/10.1136/vr.162.16.505

Nieto, D., Aramouni, M., Sibila, M., Fraile, L., Kekarainen, T., Segalés, J., 2012. Lack of effect of piglet vaccination against Porcine circovirus type 2 (PCV2) on serum viral loads of Torque teno sus virus 2 (TTSuV2). Vet. Microbiol. 157, 8–12. https://doi.org/10.1016/j.vetmic.2011.11.028

Nieto, D., Kekarainen, T., Aramouni, M., Segalés, J., 2013. Torque teno sus virus 1 and 2 distribution in tissues of porcine circovirus type 2-systemic disease affected and age-matched healthy pigs. Vet. Microbiol. 163, 364–367. https://doi.org/10.1016/j.vetmic.2013.01.005

Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., Mayumi, M., 1997. A Novel DNA Virus (TTV) Associated with Elevated Transaminase Levels in Posttransfusion Hepatitis of Unknown Etiology. Biochem. Biophys. Res. Commun. 241, 92–97. https://doi.org/https://doi.org/10.1006/bbrc.1997.7765

O'Connor, B., Gauvreau, H., West, K., Bogdan, J., Ayroud, M., Clark, E.G., Konoby, C., Allan, G., Ellis, J.A., 2001. Multiple porcine circovirus 2-associated abortions and reproductive failure in a multisite

swine production unit. Can. Vet. J. 42, 551–553.

Oliver-Ferrando, S., Segalés, J., López-Soria, S., Callén, A., Merdy, O., Joisel, F., Sibila, M., 2016. Evaluation of natural porcine circovirus type 2 (PCV2) subclinical infection and seroconversion dynamics in piglets vaccinated at different ages. Vet. Res. 47, 121. https://doi.org/10.1186/s13567-016-0405-2

Olvera, A., Sibila, M., Calsamiglia, M., Segalés, J., Domingo, M., 2004. Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. J. Virol. Methods 117, 75–80. https://doi.org/10.1016/j.jviromet.2003.12.007

Opriessnig, T., Halbur, P.G., 2012. Concurrent infections are important for expression of porcine circovirus associated disease. Virus Res. 164, 20–32. https://doi.org/10.1016/j.virusres.2011.09.014

Oropeza-Moe, M., Oropeza Delgado, A.J., Framstad, T., 2017. Porcine circovirus type 2 associated reproductive failure in a specific pathogen free (SPF) piglet producing herd in Norway: a case report. Porc. Heal. Manag. 3, 25. https://doi.org/10.1186/s40813-017-0072-3

Palinski, R., Piñeyro, P., Shang, P., Yuan, F., Guo, R., Fang, Y., Byers, E., Hause, B.M., 2017. A Novel Porcine Circovirus Distantly Related to Known Circoviruses Is Associated with Porcine Dermatitis and Nephropathy Syndrome and Reproductive Failure. J. Virol. 91, e01879-16. https://doi.org/10.1128/JVI.01879-16

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y.-L., Wang, L., Wang, D., Song, Y., Zhang, X., Huang, Y.-W., 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. Vet. Microbiol. 211, 15–21.

https://doi.org/https://doi.org/10.1016/j.vetmic.2017.09.020

Patterson, A.R., Opriessnig, T., 2010. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). Anim. Heal. Res. Rev. 11, 217–234. https://doi.org/DOI: 10.1017/S1466252310000162

P**erez, A.M., Davies, P.R., Goodell, C.K., Holtkamp, D.J., Mondaca-Fernández, E., Poljak, Z., Tousignant, S.J., Valdes-Donoso, P., Zimmerman, J.J., Morrison, R.B.,** 2015. Lessons learned and knowledge gaps about the epidemiology and control of porcine reproductive and respiratory syndrome virus in North America. J. Am. Vet. Med. Assoc. 246, 1304–1317. https://doi.org/10.2460/javma.246.12.1304

Phan, T.G., Giannitti, F., Rossow, S., Marthaler, D., Knutson, T., Li, L., Deng, X., Resende, T., Vannucci, F., Delwart, E., 2016. Detection of a novel circovirus PCV3 in pigs with cardiac and multi-systemic inflammation. Virol. J. https://doi.org/10.1186/s12985-016-0642-z

P**han, T.G., Giannitti, F., Rossow, S., Marthaler, D., Knutson, T.P., Li, L., Deng, X., Resende, T., Vannucci, F., Delwart, E.,** 2017. Erratum to: Detection of a novel circovirus PCV3 in pigs with cardiac and multi-systemic inflammation. Virol. J. 14, 87. https://doi.org/10.1186/s12985-017-0756-y

Phan, T.G., Mori, D., Deng, X., Rajidrajith, S., Ranawaka, U., Ng, T.F.F., Bucardo-Rivera, F., Orlandi, P., Ahmed, K., Delwart, E., 2015. Small viral genomes in unexplained cases of human encephalitis, diarrhea, and in untreated sewage. Virology 482, 98–104. https://doi.org/10.1016/j.virol.2015.03.011

P**ostel, A., Hansmann, F., Baechlein, C., Fischer, N., Alawi, M., Grundhoff, A., Derking, S., Tenhündfeld, J., Pfankuche, V.M., Herder, V., Baumgärtner, W., Wendt, M., Becher, P.,** 2016. Presence

of atypical porcine pestivirus (APPV) genomes in newborn piglets correlates with congenital tremor. Sci. Rep. 6, 27735. https://doi.org/10.1038/srep27735

Pringle, C.R., 1999. Virus Taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. Arch. Virol. 144, 2065–2070. https://doi.org/10.1007/s007050050728

Quintana, J., Balasch, M., Segalés, J., Calsamiglia, M., Rodríguez-Arrioja, G.M., Plana-Durán, J., Domingo, M., 2002. Experimental inoculation of porcine circoviruses type 1 (PCV1) and type 2 (PCV2) in rabbits and mice. Vet. Res. 33, 229–237. https://doi.org/10.1051/vetres:2002011

Quintana, J., Segalés, J., Calsamiglia, M., Domingo, M., 2006. Detection of porcine circovirus type 1 in commercial pig vaccines using polymerase chain reaction. Vet. J. 171, 570–573. https://doi.org/10.1016/j.tvjl.2004.12.008

Ramírez-Mendoza, H., Castillo-Juárez, H., Hernández, J., Correa, P., Segalés, J., 2009. Retrospective serological survey of Porcine circovirus-2 infection in Mexico. Can. J. Vet. Res. 73, 21–24.

Resendes, A.R., Majó, N., van den Ingh, T.S.G.A.M., Mateu, E., Domingo, M., Calsamiglia, M., Segalés, J., 2011. Apoptosis in postweaning multisystemic wasting syndrome (PMWS) hepatitis in pigs naturally infected with porcine circovirus type 2 (PCV2). Vet. J. 189, 72–76. https://doi.org/10.1016/j.tvjl.2010.06.018

Ritchie, B.W., Niagro, F.D., Lukert, P.D., Steffens, W.L., Latimer, K.S., 1989. Characterization of a new virus from cockatoos with psittacine beak and feather disease. Virology 171, 83–88. https://doi.org/10.1016/0042-6822(89)90513-8

Rodriguez-Arrioja, G.M., Segales, J., Calsamiglia, M., Resendes, A.R.,

Balasch, M., Plana-Duran, J., 2002. Dynamics of porcine circovirus type 2 infection in a herd of pigs with postweaning multisystemic wasting syndrome. Am J Vet Res. 63. https://doi.org/10.2460/ajvr.2002.63.354

Rodríguez-Arrioja, G.M., Segalés, J., Rosell, C., Rovira, A., Pujols, J., Plana-Durán, J., Domingo, M., 2003. Retrospective study on porcine circovirus type 2 infection in pigs from 1985 to 1997 in Spain. J. Vet. Med. Ser. B 50, 99–101. https://doi.org/10.1046/j.1439-0450.2003.00621.x

Roossinck, M.J., Martin, D.P., Roumagnac, P., 2015. Plant Virus Metagenomics: Advances in Virus Discovery. Phytopathology 105, 716–727. https://doi.org/10.1094/PHYTO-12-14-0356-RVW

Rosario, K., Breitbart, M., Harrach, B., Segalés, J., Delwart, E., Biagini, P., Varsani, A., 2017. Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. Arch Virol 162. https://doi.org/10.1007/s00705-017-3247-y

Rosario, K., Duffy, S., Breitbart, M., 2012. A field guide to eukaryotic circular single-stranded DNA viruses: insights gained from metagenomics. Arch. Virol. 157, 1851–1871. https://doi.org/10.1007/s00705-012-1391-y

Rose, N., Opriessnig, T., Grasland, B., Jestin, A., 2012. Epidemiology and transmission of porcine circovirus type 2 (PCV2). Virus Res. 164, 78–89. https://doi.org/10.1016/j.virusres.2011.12.002

Rosell, C., Segalés, J., Plana-Durán, J., Balasch, M., Rodríguez-Arrioja, G.M., Kennedy, S., 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J Comp Pathol 120. https://doi.org/10.1053/jcpa.1998.0258

Rovira, A., Balasch, M., Segalés, J., García, L., Plana-Durán, J., Rosell, C., Ellerbrok, H., Mankertz, A., Domingo, M., 2002. Experimental Inoculation of Conventional Pigs with Porcine Reproductive and Respiratory Syndrome Virus and Porcine Circovirus 2. J. Virol. 76, 3232–3239. https://doi.org/10.1128/JVI.76.7.3232-3239.2002

Ruiz-Fons, F., Segalés, J., Gortázar, C., 2008. A review of viral diseases of the European wild boar: Effects of population dynamics and reservoir rôle. Vet. J. 176, 158–169. https://doi.org/10.1016/j.tvjl.2007.02.017

Sanchez, R.E., Meerts, P., Nauwynck, H.J., Ellis, J.A., Pensaert, M.B., 2004. Characteristics of Porcine Circovirus—2 Replication in Lymphoid Organs of Pigs Inoculated in Late Gestation or Postnatally and Possible Relation to Clinical and Pathological Outcome of Infection. J. Vet. Diagnostic Investig. 16, 175–185. https://doi.org/10.1177/104063870401600301

Sanchez, R.E., Meerts, P., Nauwynck, H.J., Pensaert, M.B., 2003. Change of porcine circovirus 2 target cells in pigs during development from fetal to early postnatal life. Vet. Microbiol. 95, 15–25. https://doi.org/10.1016/S0378-1135(03)00120-2

Sanchez, R.E., Nauwynck, H.J., McNeilly, F., Allan, G.M., Pensaert, M.B., 2001. Porcine circovirus 2 infection in swine foetuses inoculated at different stages of gestation. Vet Microbiol. 83. https://doi.org/10.1016/S0378-1135(01)00425-4

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

Saraiva, G.L., Vidigal, P.M.P., Fietto, J.L.R., Bressan, G.C., Silva Júnior, A., de Almeida, M.R., 2018. Evolutionary analysis of Porcine circovirus 3 (PCV3) indicates an ancient origin for its current strains and a worldwide dispersion. Virus Genes 54, 376–384.

https://doi.org/10.1007/s11262-018-1545-4

Sarno, M., Aquino, M., Pimentel, K., Cabral, R., Costa, G., Bastos, F., Brites, C., 2016. Progressive lesions of central nervous system in microcephalic fetuses with suspected congenital Zika virus syndrome. Ultrasound Obstet. Gynecol. 50, 717–722. https://doi.org/10.1002/uog.17303

Sato, G., Kawashima, T., Kiuchi, M., Tohya, Y., 2015. Novel cyclovirus detected in the intestinal contents of Taiwan squirrels (Callosciurus erythraeus thaiwanensis). Virus Genes 51, 148–151. https://doi.org/10.1007/s11262-015-1217-6

Schlosser, J., Vina-Rodriguez, A., Fast, C., Groschup, M.H., Eiden, M., 2015. Chronically infected wild boar can transmit genotype 3 hepatitis E virus to domestic pigs. Vet. Microbiol. 180, 15–21. https://doi.org/10.1016/j.vetmic.2015.08.022

Schulze, C., Neumann, G., Hlinak, A., Segales, J., Calsamiglia, M., Domingo, M., 2004. Identification of postweaning multisystemic wasting syndrome in European wild boar (Sus scrofa). Vet. Rec. 154, 694 LP-696.

Segalés, J., 2015. Best practice and future challenges for vaccination against porcine circovirus type 2. Expert Rev. Vaccines 14, 473–487. https://doi.org/10.1586/14760584.2015.983084

Segalés, J., 2012. Porcine circovirus type 2 (PCV2) infections: Clinical signs, pathology and laboratory diagnosis. Virus Res. 164, 10–19. https://doi.org/10.1016/j.virusres.2011.10.007

Segalés, J., Allan, G.M., Domingo, M., 2012. Porcine Circoviruses, in: Diseases of Swine.

Segalés, J., Allan, G.M., Domingo, M., 2005a. Porcine circovirus diseases. Anim Heal. Res Rev 6. https://doi.org/10.1079/AHR2005106

Segalés, J., Calsamiglia, M., Olvera, A., Sibila, M., Badiella, L., Domingo, M., 2005b. Quantification of porcine circovirus type 2 (PCV2) DNA in serum and tonsillar, nasal, tracheo-bronchial, urinary and faecal swabs of pigs with and without postweaning multisystemic wasting syndrome (PMWS). Vet. Microbiol. 111, 223–229. https://doi.org/10.1016/j.vetmic.2005.10.008

Segalés, J., Domingo, M., 2002. Postweaning mulstisystemic wasting syndrome (PMWS) in pigs. A review. Vet. Q. 24, 109–124. https://doi.org/10.1080/01652176.2002.9695132

Segalés, J., Kekarainen, T., Cortey, M., 2013. The natural history of porcine circovirus type 2: From an inoffensive virus to a devastating swine disease? Vet Microbiol. 165. https://doi.org/10.1016/j.vetmic.2012.12.033

Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grassland, B., Baekbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. Vet. Rec. 162, 867 LP-868.

Segalés, J., Piella, J., Marco, E., Mateu-de-Antonio, E.M., Espuña, E., Domingo, M., 1998. Porcine dermatitis and nephropathy syndrome in Spain. Vet. Rec. 142, 483 LP-486.

Segalés, J., Rosell, C., Domingo, M., 2004. Pathological findings associated with naturally acquired porcine circovirus type 2 associated disease. Vet. Microbiol. 98, 137–149. https://doi.org/10.1016/j.vetmic.2003.10.006

Shen, H., Liu, X., Zhang, P., Wang, L., Liu, Y., Zhang, L., Liang, P., Song, C., 2017. Genome characterization of a porcine circovirus type 3 in South China. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12639

Shi, M., Lemey, P., Singh Brar, M., Suchard, M.A., Murtaugh, M.P., Carman, S., D'Allaire, S., Delisle, B., Lambert, M.-È., Gagnon, C.A., Ge, L., Qu, Y., Yoo, D., Holmes, E.C., Chi-Ching Leung, F., 2013. The spread of Type 2 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in North America: A phylogeographic approach. Virology 447, 146–154. https://doi.org/10.1016/j.virol.2013.08.028

Shibata, I., Okuda, Y., Kitajima, K., Asai, T., 2006. Shedding of Porcine Circovirus into Colostrum of Sows. J. Vet. Med. Ser. B 53, 278–280. https://doi.org/10.1111/j.1439-0450.2006.00953.x

Shibata, I., Tsuda, T., Mori, M., Ono, M., Sueyoshi, M., Uruno, K., 2000. Isolation of porcine epidemic diarrhea virus in porcine cell cultures and experimental infection of pigs of different ages. Vet. Microbiol. 72, 173–182. https://doi.org/10.1016/S0378-1135(99)00199-6

Shin, M.-K., Yoon, S.H., Kim, M.H., Lyoo, Y.S., Suh, S.W., Yoo, H.S., 2015. Assessing PCV2 antibodies in field pigs vaccinated with different porcine circovirus 2 vaccines using two commercial ELISA systems. J. Vet. Sci. 16, 25–29. https://doi.org/10.4142/jvs.2015.16.1.25

Sibila, M., Calsamiglia, M., Segales, J., Blanchard, P., Badiella, L., Dimna, M., 2004. Use of a polymerase chain reaction assay and an ELISA to monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. Am J Vet Res. 65. https://doi.org/10.2460/ajvr.2004.65.88

Smits, S.L., Zijlstra, E.E., van Hellemond, J.J., Schapendonk, C.M.E., Bodewes, R., Schürch, A.C., Haagmans, B.L., Osterhaus, A.D.M.E., 2013. Novel Cyclovirus in Human Cerebrospinal Fluid, Malawi, 2010–2011. Emerg. Infect. Dis. 19, 1511–1513. https://doi.org/10.3201/eid1909.130404

Song, D., Chen, Y., Peng, Q., Huang, D., Zhang, T., Huang, T., Zhang, F.,

Zhou, X., Tang, Y., 2014. Full-Length Genome Sequence of a Variant Porcine Epidemic Diarrhea Virus Strain, CH/GDZQ/2014, Responsible for a Severe Outbreak of Diarrhea in Piglets in Guangdong, China, 2014. Genome Announc. 2, e01239-14. https://doi.org/10.1128/genomeA.01239-14

Stadejek, T., Woźniak, A., Miłek, D., Biernacka, K., 2017. First detection of porcine circovirus type 3 on commercial pig farms in Poland. Transbound. Emerg. Dis. 64, 1350–1353. https://doi.org/10.1111/tbed.12672

Stevenson, G.W., Kiupel, M., Mittal, S.K., Choi, J., Latimer, K.S., Kanitz, C.L., 2001. Tissue Distribution and Genetic Typing of Porcine Circoviruses in Pigs with Naturally Occurring Congenital Tremors. J. Vet. Diagnostic Investig. 13, 57–62. https://doi.org/10.1177/104063870101300111

Sun, J., Wei, L., Lu, Z., Mi, S., Bao, F., Guo, H., Tu, C., Zhu, Y., Gong, W., 2018. Retrospective study of porcine circovirus 3 infection in China. Transbound. Emerg. Dis. 65, 607–613. https://doi.org/10.1111/tbed.12853

Sun, R.-Q., Cai, R.-J., Chen, Y.-Q., Liang, P.-S., Chen, D.-K., Song, C.-X., 2012. Outbreak of Porcine Epidemic Diarrhea in Suckling Piglets, China. Emerg. Infect. Dis. 18, 161–163. https://doi.org/10.3201/eid1801.111259

Sun, S.-Q., Guo, H.-C., Sun, D.-H., Yin, S.-H., Shang, Y.-J., Cai, X.-P., Liu, X.-T., 2010. Development and validation of an ELISA using a protein encoded by ORF2 antigenic domain of porcine circovirus type 2. Virol. J. 7, 274. https://doi.org/10.1186/1743-422X-7-274

Taubenberger, J.K., Morens, D.M., 2006. Influenza Revisited. Emerg. Infect. Dis. 12, 1–2. https://doi.org/10.3201/eid1201.051442

Taylor, L.H., Latham, S.M., Woolhouse, M.E., 2001. Risk factors for human

disease emergence. Philos. Trans. R. Soc. B Biol. Sci. 356, 983–989. https://doi.org/10.1098/rstb.2001.0888

Thimmasandra Narayanappa, A., Sooryanarain, H., Deventhiran, J., Cao, D., Ammayappan Venkatachalam, B., Kambiranda, D., LeRoith, T., Heffron, C.L., Lindstrom, N., Hall, K., Jobst, P., Sexton, C., Meng, X.-J., Elankumaran, S., 2015. A Novel Pathogenic Mammalian Orthoreovirus from Diarrheic Pigs and Swine Blood Meal in the United States. MBio 6, e00593-15. https://doi.org/10.1128/mBio.00593-15

Ticó, G., Segalés, J., Martínez, J., 2013. The blurred border between porcine circovirus type 2-systemic disease and porcine respiratory disease complex. Vet. Microbiol. 163, 242–247. https://doi.org/10.1016/j.vetmic.2013.01.001

Tischer, I., Bode, L., Peters, D., Pociuli, S., Germann, B., 1995a. Distribution of antibodies to porcine circovirus in swine populations of different breeding farms. Arch. Virol. 140, 737–743. https://doi.org/10.1007/BF01309961

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. Nature 295, 64–66.

Tischer, I., Mields, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. 91, 271–276. https://doi.org/10.1007/BF01314286

Tischer, I., Peters, D., Pociuli, S., 1995b. Occurrence and role of an early antigen and evidence for transforming ability of porcine circovirus. Arch. Virol. 140, 1799–1816. https://doi.org/10.1007/BF01384343

Tischer, I., Peters, D., Rasch, R., Pociuli, S., 1987. Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. Arch. Virol. 96, 39–57. https://doi.org/10.1007/BF01310989

Tischer, I., Rasch, R., Tochtermann, G., 1974. Characterization of papovavirus-and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl. Bakteriol. Orig. A. 226, 153—167.

Tochetto, C., Lima, D.A., Varela, A.P.M., Loiko, M.R., Paim, W.P., Scheffer, C.M., Herpich, J.I., Cerva, C., Schmitd, C., Cibulski, S.P., Santos, A.C., Mayer, F.Q., Roehe, P.M., 2017. Full-Genome Sequence of Porcine Circovirus type 3 recovered from serum of sows with stillbirths in Brazil. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12735

Todd, D., 2000. Circoviruses: Immunosuppressive threats to avian species: A review. Avian Pathol. 29, 373–394. https://doi.org/10.1080/030794500750047126

Verreault, D., Létourneau, V., Gendron, L., Massé, D., Gagnon, C.A., Duchaine, C., 2010. Airborne porcine circovirus in Canadian swine confinement buildings. Vet. Microbiol. 141, 224–230. https://doi.org/10.1016/j.vetmic.2009.09.013

Vincent, I.E., Carrasco, C.P., Guzylack-Piriou, L., Herrmann, B., McNeilly, F., Allan, G.M., 2005. Subset-dependent modulation of dendritic cell activity by circovirus type 2. Immunology. 115. https://doi.org/10.1111/j.1365-2567.2005.02165.x

Wang, C., Pang, V.F., Lee, F., Huang, T.-S., Lee, S.-H., Lin, Y.-J., Lin, Y.-L., Lai, S.-S., Jeng, C.-R., 2013. Prevalence and genetic variation of porcine circovirus type 2 in Taiwan from 2001 to 2011. Res. Vet. Sci. 94, 789–795. https://doi.org/10.1016/j.rvsc.2012.11.019

Wang, L., Byrum, B., Zhang, Y., 2014. Detection and Genetic Characterization of Deltacoronavirus in Pigs, Ohio, USA, 2014. Emerg. Infect. Dis. 20, 1227–1230. https://doi.org/10.3201/eid2007.140296

West, K.H., Bystrom, J.M., Wojnarowicz, C., Shantz, N., Jacobson, M., Allan, G.M., Haines, D.M., Clark, E.G., Krakowka, S., McNeilly, F.,

Konoby, C., Martin, K., Ellis, J.A., 1999. Myocarditis and Abortion Associated with Intrauterine Infection of Sows with Porcine Circovirus 2. J. Vet. Diagnostic Investig. 11, 530–532. https://doi.org/10.1177/104063879901100608

World Health Organization-WHO. Retrieved from https://www.who.int/csr/disease/ebola/en/

World Health Organization-WHO. Retrieved from https://www.who.int/csr/disease/zika-virus/en/

Wiederkehr, D.D., Sydler, T., Buergi, E., Haessig, M., Zimmermann, D., Pospischil, A., Brugnera, E., Sidler, X., 2009. A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. Vet. Microbiol. 136, 27–35. https://doi.org/10.1016/j.vetmic.2008.10.028

Witzany, G., 2006. Natural Genome-Editing Competences of Viruses. Acta Biotheor. 54, 235–253. https://doi.org/10.1007/s10441-006-9000-7

Wood, D.E., Salzberg, S.L., 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 15, R46–R46. https://doi.org/10.1186/gb-2014-15-3-r46

Woolhouse, M.E.J., Taylor, L.H., Haydon, D.T., 2001. Population Biology of Multihost Pathogens. Science. 292, 1109–1112. https://doi.org/10.1126/science.1059026

Wu, Z., Ren, X., Yang, L., Hu, Y., Yang, J., He, G., Zhang, J., Dong, J., Sun, L., Du, J., Liu, L., Xue, Y., Wang, J., Yang, F., Zhang, S., Jin, Q., 2012. Virome Analysis for Identification of Novel Mammalian Viruses in Bat Species from Chinese Provinces. J. Virol. 86, 10999–11012. https://doi.org/10.1128/JVI.01394-12

Xiao, C.T., Halbur, P.G., Opriessnig, T., 2015. Global molecular genetic analysis of porcine circovirus type 2 (PCV2) sequences confirms the

presence of four main PCV2 genotypes and reveals a rapid increase of PCV2d. J. Gen. Virol. https://doi.org/10.1099/vir.0.000100

Xu, P.-L., Zhang, Y., Zhao, Y., Zheng, H.-H., Han, H.-Y., Zhang, H.-X., Chen, H.-Y., Yang, M.-F., Zheng, L.-L., 2018. Detection and phylogenetic analysis of porcine circovirus type 3 in central China. Transbound. Emerg. Dis. 0. https://doi.org/10.1111/tbed.12920

Ye, X., Berg, M., Fossum, C., Wallgren, P., Blomström, A.-L., 2018. Detection and genetic characterisation of porcine circovirus 3 from pigs in Sweden. Virus Genes 54, 466–469. https://doi.org/10.1007/s11262-018-1553-4

Yu, S., Opriessnig, T., Kitikoon, P., Nilubol, D., Halbur, P.G., Thacker, E., 2007. Porcine circovirus type 2 (PCV2) distribution and replication in tissues and immune cells in early infected pigs. Vet. Immunol. Immunopathol. 115, 261–272. https://doi.org/10.1016/j.vetimm.2006.11.006

Yuzhakov, A.G., Raev, S.A., Alekseev, K.P., Grebennikova, T. V, Verkhovsky, O.A., Zaberezhny, A.D., Aliper, T.I., 2018. First detection and full genome sequence of porcine circovirus type 3 in Russia. Virus Genes 54, 608–611. https://doi.org/10.1007/s11262-018-1582-z

Zhai, S.-L., Zhou, X., Zhang, H., Hause, B.M., Lin, T., Liu, R., Chen, Q.-L., Wei, W.-K., Lv, D.-H., Wen, X.-H., Li, F., Wang, D., 2017. Comparative epidemiology of porcine circovirus type 3 in pigs with different clinical presentations. Virol. J. 14, 222. https://doi.org/10.1186/s12985-017-0892-4

Zhang, D., He, K., Wen, L., Fan, H., 2015. Genetic and phylogenetic analysis of a new porcine circovirus type 2 (PCV2) strain in China. Arch. Virol. 160, 3149–3151. https://doi.org/10.1007/s00705-015-2615-8

Zhang, J., Liu, Z., Zou, Y., Zhang, N., Wang, D., Tu, D., Yang, L., Deng,

Z., Yang, Y., Jiang, P., Wang, N., 2017. First molecular detection of porcine circovirus type 3 in dogs in China. Virus Genes. https://doi.org/10.1007/s11262-017-1509-0

Zhang, W., Li, L., Deng, X., Kapusinszky, B., Pesavento, P.A., Delwart, E., 2014. Faecal virome of cats in an animal shelter. J. Gen. Virol. 95, 2553–2564. https://doi.org/10.1099/vir.0.069674-0

Zhao, D., Wang, X., Gao, Q., Huan, C., Wang, W., Gao, S., Liu, X., 2018. Retrospective survey and phylogenetic analysis of porcine circovirus type 3 in Jiangsu province, China, 2008 to 2017. Arch. Virol. https://doi.org/10.1007/s00705-018-3870-2

Zheng, S., Shi, J., Wu, X., Peng, Z., Xin, C., Zhang, L., Liu, Y., Gao, M., Xu, S., Han, H., Yu, J., Sun, W., Cong, X., Li, J., Wang, J., 2018. Presence of Torque teno sus virus 1 and 2 in porcine circovirus 3-positive pigs. Transbound. Emerg. Dis. 65, 327–330. https://doi.org/10.1111/tbed.12792

Zheng, S., Wu, X., Zhang, L., Xin, C., Liu, Y., Shi, J., Peng, Z., Xu, S., Fu, F., Yu, J., Sun, W., Xu, S., Li, J., Wang, J., 2017. The occurrence of porcine circovirus 3 without clinical infection signs in Shandong Province. Transbound. Emerg. Dis. 64, 1337–1341. https://doi.org/10.1111/tbed.12667

Zou, Y., Zhang, N., Zhang, J., Zhang, S., Jiang, Y., Wang, D., Tan, Q., Yang, Y., Wang, N., 2018. Molecular detection and sequence analysis of porcine circovirus type 3 in sow sera from farms with prolonged histories of reproductive problems in Hunan, China. Arch. Virol. https://doi.org/10.1007/s00705-018-3914-7