Morphologic studies of the pathogenesis of naturally occurring porcine circovirus type 2 diseases in pigs

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Morphologic studies of the pathogenesis of naturally occurring porcine circovirus type 2 diseases in pigs

PhD thesis

Mette Sif Hansen

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Preface

This thesis is based upon work performed at The Department of Veterinary Disease Biology, Faculty of Life Sciences (LIFE), University of Copenhagen, Denmark and at The Department of Veterinary Diagnostics and Research, The National Veterinary Institute, Technical University of Denmark (DTU).

So many people have helped and supported me during my years as a Ph.D. student, and those I would like to thank, especially the following have had a great influence on the outcome of my thesis:

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Mette Sif Hansen, Strøby Egede, 2009

List of abbreviations

AEC	3-amino-9-ethylcarbazole
A. pleuropneumoniae	*Actinobacillus pleuropneumoniae*
ASBP	Acute suppurative bronchopneumonia
BALT	Bronchus-associated lymphoid tissue
BFDV	Beak and feather disease virus
BM	Bone marrow
CaCV	Canary circovirus
CAV	Chicken anaemia virus
CBP	Cranioventral bronchopneumonia
CD/CD	Caesarean-derived, colostrum-deprived
CMBP	Chronic mixed bronchopneumonia
CNBP	Chronic non-suppurative bronchopneumonia
CP	Complementary probe
CR	Crown to rump length
CSBP	Chronic suppurative bronchopneumonia
CT	Congenital tremor
DCs	Dendritic cells
DG	Days of gestation
DIG	Digoxigenin
DKF	Danish key figures
ds	Double-stranded
DTU	Technical University of Denmark
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMCV	Encephalomyocarditis virus
FA	Foetal age in days
FAEM	Fat associated eosinophilic material
FDC	Follicular dendritic cell
FDC-SP	Follicular dendritic cell secreted protein
GoCV	Goose circovirus

HE	Hematoxylin and eosin
HIV	Humane immunodeficiency virus type 1
HP & BC	Haematopoetic precursors and blood cells
H. parasuis	*Haemophilus parasuis*
IF	Immunofluorescence
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IPP	Ileal Peyer's patch
ISH	In situ hybridisation
KLH/ICFA	Keyhole limpet hemocyanin emulsified in incomplete Freund´s adjuvant
LIFE	Faculty of Life Sciences
mAb	Monoclonal antibody
M:E	Myeloid to erythroid ratio
MGC	Multinucleated giant cells
MHCII	Major histocompatibility complex class II
M. hyopneumonia	*Mycoplasma hyopneumoniae*
M. hyorhinis	*Mycoplasma hyorhinis*
mRNA	Messenger RNA
NaOH	Sodium hydroxide
NSS	Normal swine serum
OD% >44	Optical densities above 44
ORF	Open reading frame
PCF	Pleura cavity fluid
PCMV	Porcine cytomegalovirus
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV1	Porcine circovirus type 1
PCV2	Porcine circovirus type 2
PCVDs	Porcine circovirus diseases
PDNS	Porcine dermatitis and nephropathy syndrome
PI	Post inoculation
PiCV	Pigeon circovirus

P. multocida	*Pasteurella multocida*
PMWS	Postweaning multisystemic wasting syndrome
PNP	Proliferative and necrotizing pneumonia
PPV	Porcine parvovirus
PRCV	Porcine respiratory coronavirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
RF	Replicative form
RFP	Replicative form probe
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SIV	Swine influenza virus
SMBP	Subacute mixed bronchopneumonia
SNBP	Subacute non-suppurative bronchopneumonia
SPF	Specific pathogen free
ss	Single-stranded
SSBP	Subacute suppurative bronchopneumonia
S. suis	*Streptococcus suis*
STP	Society of Toxicologic Pathology
TBS	Tris-buffered saline
$TCID_{50}\,ml^{-1}$	50% tissue culture infectious dose per millilitre
TTV	Torque teno virus
TTVM	Torque teno mini virus

Summary

This Ph.D. thesis presents different aspects of porcine circovirus diseases (PCVDs), based upon work performed at The Faculty of Life Sciences, University of Copenhagen, Denmark and at The National Veterinary Institute, Technical University of Denmark.

The introduction, *chapter 1*, briefly presents the background and objectives of this study.

Chapter 2 describes different aspects of porcine circovirus type 2 (PCV2), including molecular biology, genotypes, other closely related circoviruses and also the pathogenesis of PCV2 infections. Furthermore, the different porcine circovirus diseases (PCVDs) and experimental models of PCVDs are reviewed.

In *chapter 3*, the results achieved by the research are presented as four manuscripts (I-IV):

- Manuscript I is focussed on the presence of PCV2 and viral replication, in the bone marrow and thymus of experimentally infected pigs and pigs naturally affected by postweaning multisystemic wasting syndrome (PMWS). By in situ hybridization (ISH) virus and viral replication was detected, mainly in histiocyte-like cells, in both organs of the PMWS-affected pigs. However, there was no evidence that bone marrow or thymus is especially important in regard of PCV2 replication.

- In manuscript II the occurrence and tissue distribution of PCV2 is examined, by immunohistochemistry (IHC) of lungs, kidneys, lymph nodes and spleen from slaughtered Danish finishing pigs with or without bronchopneumonia. Detection of PCV2 could not be related to lesions or PCVD. The virus was mainly detected in lymph nodes in association with follicular dendritic cells, which were identified by IHC. Whether this finding represents subclinical or persistent infection, or a status after PCV2 infection with innocent storage of antigen, remains unclear.

- Manuscript III focuses on the progression and diagnostics of an outbreak of naturally occurring PCV2 associated reproductive failure in a Danish herd. In stillborn and

mummified foetuses PCV2 was detected mainly in the heart and bone marrow, by IHC, and replication was detected in myocardiocytes, by ISH. The study showed that measurements of total immunoglobulin G in foetuses are not very reliable as indication of intrauterine infection with PCV2. Furthermore, real-time polymerase chain reaction proved to be better for the detection of PCV2 in infected foetuses, compared to IHC, as PCV2 only was detected early in the outbreak by the latter method.

- Manuscript IV is an update on the pathology and pathogens present in porcine respiratory disease complex (PRDC) in Danish finishing pigs. Multiple pathogens were detected, including PCV2, confirming the previously reported pathogen profile and multifactorial aetiology of PRDC. The histopathology was very variable and no clear-cut associations were detected between specific pathogens and histological lesions.

In *chapter 4*, the achieved results are discussed and the future perspectives are addressed, and the thesis is closed with a summary of the drawn conclusions.

Sammendrag (Danish summary)

I ph.d. afhandlingen præsenteres forskellige aspekter af porcin circovirus type 2 (PCV2) associerede sygdomme, baseret på arbejde udført ved Institut for Veterinær Sygdomsbiologi, Det Biovidenskabelige Fakultet for Fødevarer, Veterinærmedicin og Naturressourcer, Københavns Universitet og Veterinærinstituttet, Danmarks Tekniske Universitet.

Introduktionen, *kapitel 1*, giver en kort præsentation af baggrunden og formålene med dette studie.

Kapitel 2, beskriver PCV2´s molekylære biologi, genotyper og andre nært beslægtede circovirusser, samt patogenese vedrørende PCV2 infektionen. Yderligere gennemgås de forskellige PCV2 associerede sygdomme og eksperimentelle studier relateret hertil.

I *kapitel 3*, præsenteres de opnåede forskningsresultater i form af fire manuskripter (I-IV):

- Manuskript I fokuserer på tilstedeværelsen af PCV2, samt viral replikation i knoglemarv og thymus hos eksperimentelt inficerede grise og grise med spontant forekommende "postweaning multisystemic wasting syndrome" (PMWS). Ved hjælp af in situ hybridisering (ISH) blev virus og virus replikation påvist, primært i makrofag-lignende celler, i begge organer fra de PMWS-afficerede grise. Resultaterne tyder dog ikke på at knoglemarv eller thymus er af speciel betydning for PCV2 replikationen.

- I manuskript II undersøges tilstedeværelse og udbredelsen af PCV2 vha. immunhistokemi (IHC), i lunger, nyre, lymfeknuder og milt fra danske slagtesvin med eller uden bronchopneumoni. Fund af PCV2 kunne ikke forbindes med læsioner eller PCV2 associeret sygdom. Virus fandtes primært i lymfeknuder associeret til follikulære denritiske celler, der blev identificeret vha. IHC. Hvorvidt dette fund repræsenterer subklinisk eller persisterende infektion eller status efter PCV2 infektion med harmløs lagring af antigen, er dog uvist.

- Manuskript III fokuserer på forløbet og diagnostik af PCV2 associerede reproduktions problemer, baseret på et spontant forekommende tilfælde i en dansk besætning. I dødfødte og mummificerede fostre blev PCV2 påvist, primært i hjerte og knoglemarv vha. IHC, og

replikation blev detekteret i myocardiocytter, vha. ISH. Studiet viste at det kan være tvivlsomt at bruge måling af total immunoglobulin G i fostre, til diagnostik af intrauterin infektion med PCV2. Yderligere, viste "real-time polymerase chain reaction" sig at være bedre til at detektere PCV2 i inficerede fostre, i forhold til IHC, da denne metode kun påviste PCV2 tidligt i sygdomsforløbet i besætningen.

- Manuskript IV er en opdatering af patologi og patogener, der forekommer i forbindelse med "porcine respiratory disease complex" (PRDC) i danske slagtesvin. Multiple patogener, heriblandt PCV2, blev påvist, og dette understøtter tidligere beskrivelser af PRDC pathogenprofilen og den multifaktorielle ætiologi. Histopatologien varierede meget og der fandtes ingen entydige sammenhænge mellem fund af specifikke patogener og histologiske læsioner.

Kapitel 4 indeholder en samlet diskussion og perspektivering af de opnåede resultater, og der afsluttes med en opsummering af de dragne konklusioner.

Chapter 1 Introduction

Porcine circovirus type 2 (PCV2) occurs worldwide in the swine population and besides causing subclinical infections, the virus is associated with different disease complexes of which the most prevalent and well known is postweaning multisystemic wasting syndrome (PMWS). Other important porcine circovirus diseases (PCVDs) are: PCV2 associated reproductive failure, porcine respiratory disease complex and porcine dermatitis and nephropathy syndrome. Opriessnig et al. (2007) suggested that PCVDs are separated into systemic infections, i.e. involvement of several organs as in PMWS, and local diseases, which only affects specific organ systems, e.g. lungs, reproductive organs or intestines. Some of the main features that associate PCVDs are depletion of lymphocytes and infiltration of histiocytes in the lymphoid tissues, and lymphohistiocytic infiltrations can be seen in other organs (Opriessnig et al., 2007; Segalés et al., 2004b). Since the first case of PMWS was reported in 1996 (Harding and Clark, 1997), this disease complex has been responsible for reduced swine welfare and substantial economic losses in the pig industry. Thus, the losses in Europe were estimated to 900 million Euro in 2004 (Armstrong and Bishop, 2004) and 500 million Euro in 2007 (Anonymous, 2007).

To date, much work has been carried out to elucidate different aspects of the PCV2 infection, but there are still many unexplained features, especially concerning the pathogenesis of the infection (Allan and McNeilly, 2006; Opriessnig et al., 2007). Some of the questions that still need answers, are: what is the biological background for the “sudden” emergence of PMWS, even though PCV2 already had been in the pig population for some time; why do some pigs develop clinical manifestations of PCVDs, whereas others only get subclinically infected; what causes the discrepancy between the detection of huge amounts of virus in diseased pigs, together with no or low grade of viral replication; what is the mechanism of the lymphocyte depletion observed in the lymphoid organs; what is the exact role of PCV2 in the different PCVDs; how should we further develop the treatment and prophylaxis strategies, and evaluate their effect on subclinical and clinical PCV2 infections in pigs of different ages; etc.

Objectives

The main objective of the study reported here, was to increase the knowledge of PCVDs with focus on pathogenesis and pathology. This was done by investigating systemic and local manifestations of the PCV2 infection in pigs of different ages. The specific objectives of the studies included in this thesis, were:

- To assess the role of the primary lymphoid organs during the PCV2 infection in naturally and experimentally infected pigs (manuscript I).
- To study the occurrence, distribution and significance of PCV2 antigens detected in naturally infected Danish finishing pigs at slaughter (manuscript II).
- To evaluate aspects of the pathogenesis and diagnosis of naturally occurring PCV2 associated reproductive failure (manuscript III).
- To get an update on the pathogens and pathogenesis involved in PRDC in Danish finishing pigs (manuscript IV).

Chapter 2 Background

Porcine circoviruses

In 1974, Tischer et al. (1974) reported a picornavirus-like viral contaminant of the porcine permanent kidney cell line, PK-15. Later on, this virus was named porcine circovirus (PCV) based on the circular arrangement of the viral genome (Tischer et al., 1982). Antibodies to PCV were found to be widespread in the swine population and the virus was recognized as non-pathogenic (Tischer et al., 1986). Two decades later in Europe and North America, a second porcine circovirus was isolated from pigs suffering from a wasting syndrome, which later was named postweaning multisystemic wasting syndrome (PMWS) (Allan et al., 1998; Meehan et al., 1998). Hence, the two circoviruses were named porcine circovirus type 1 (PCV1) and 2 (PCV2), respectively (Meehan et al., 1998) and were classified in the viral family *Circoviridae* (genus *Circovirus*) (Todd et al., 2005). Though, PCV2 associated disease was not recognized until the 1990s, retrospective studies have shown that the virus was present in the swine population as early as 1969 in Europe and 1985 in Canada (Opriessnig et al., 2007).

Molecular biology of porcine circoviruses

Porcine circoviruses are non-enveloped, covalently closed, circular single-stranded (ss) DNA viruses of 17 nm in diameter and are thus the smallest DNA viruses replicating autonomously in mammalian cells (Mankertz et al., 2004; Tischer et al., 1982). They replicate by a process known as rolling-circle replication, which is initiated from a stem-loop structure, a conserved nucleotide sequence (Cheung, 2007; Steinfeldt et al., 2001) (figure 1). During replication the virus goes through an intermediate double-stranded (ds) DNA form, i.e. the replicative form (RF) consisting of the viral ssDNA and the complementary strand (Steinfeldt et al., 2006; Todd et al., 2005) (figure 2). The replication process depends on nuclear DNA polymerases in the host cell, expressed during the s-phase of the growth cycle (Tischer et al., 1986; Tischer et al., 1987). The viral genome has an ambisense organization and contains two well-characterised open reading frames (ORF), which are divergently located on opposite strands of the RF (figure 1). ORF1 represents the *rep* gene and is

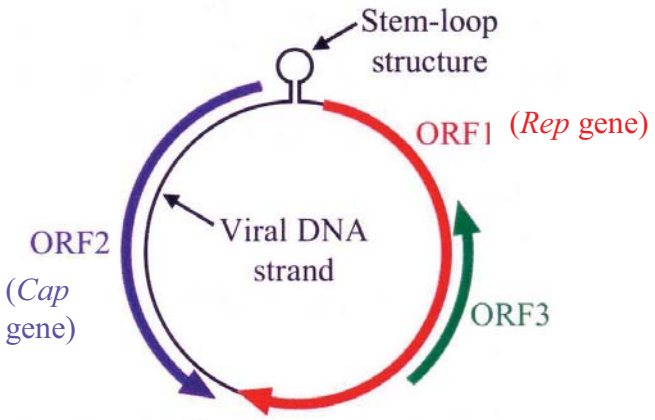

Fig. 1. Genome structure of porcine circoviruses. The circular single-stranded DNA genome is covalently closed and contains a stem-loop structure. The genome has an ambisense organization, where the open reading frames (ORF) are divergently located on opposite strands of the double-stranded replicative form. ORF1 (red) is transcribed from the viral ssDNA in a clockwise orientation, whereas ORF2 (blue) and ORF3 (green) are transcribed counter-clockwise from the complementary DNA strand. (Figure modified after Lefebvre, 2009)

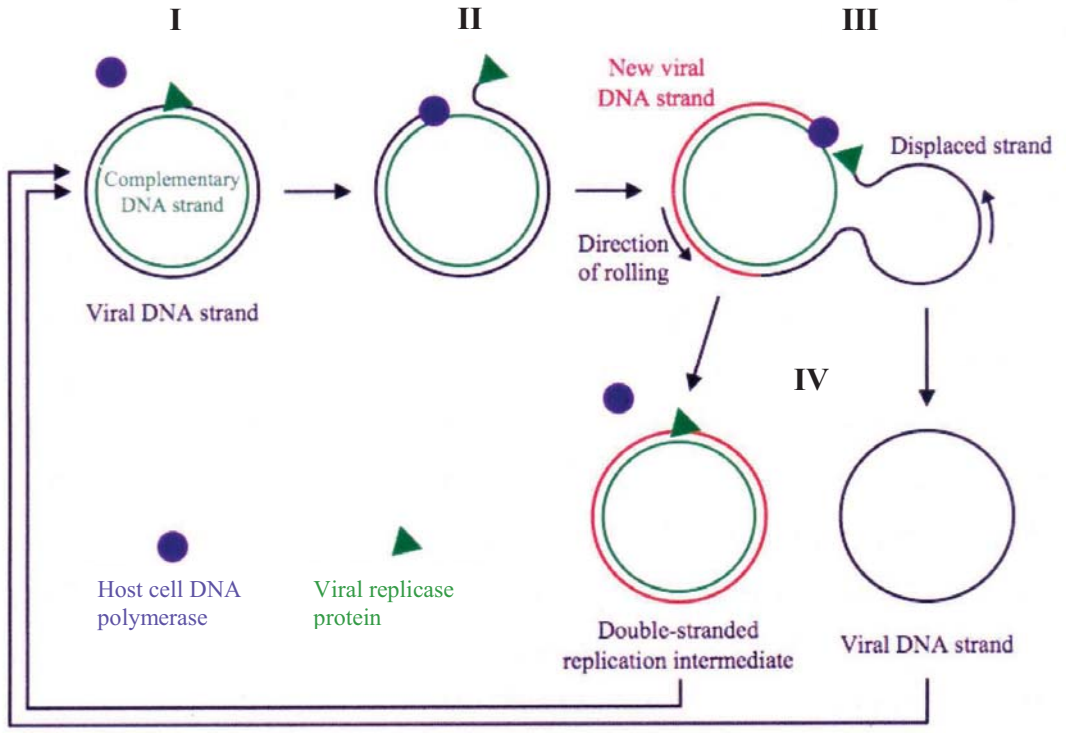

Fig. 2. Rolling-circle replication of porcine circoviruses. (I) By means of nuclear polymerases of the host cell, the single-stranded (ss) viral DNA (blue) is used as template for synthesis of a complementary DNA strand (green), hereby creating an intermediate double-stranded DNA replicative form (RF). (II) The viral replicase proteins bind to the RF and cleave the viral DNA. (III) The complementary strand is used as template for synthesis of new viral DNA (red), (IV) resulting in the release of a circular ssDNA molecule and the production of a new RF (Steinfeldt et al., 2006). (Figure modified after Lefebvre, 2009)

located on the viral ssDNA strand in a clockwise orientation, whereas ORF2 with the *cap* gene, is expressed on the complementary strand (Todd et al., 2005). Thus messenger RNA (mRNA) is transcribed from both strands of the RF (Mankertz et al., 1998). The *rep* gene encodes two major replicase proteins, which both are essential for the replication of viral DNA: the full length Rep protein; and Rep´, which is a spliced product of the *rep* gene (Cheung, 2003; Mankertz et al., 2004). The replicase proteins bind to dsDNA, but only cleaves ssDNA (Mankertz et al., 2004; Steinfeldt et al., 2001; Steinfeldt et al., 2006). The major viral structural capsid protein is encoded by the *cap* gene (Nawagitgul et al., 2000). On the complementary strand of the RF, a third ORF (ORF3) (Liu et al., 2005; Morozov et al., 1998), which might be involved in the viral pathogenesis, has been characterised recently (Karuppannan et al., 2009; Liu et al., 2005) (figure 1).

Porcine circovirus genotypes

Nucleotide sequence identity between PCV1 and PCV2 isolates is 68-76% (Cheung and Bolin, 2002), whereas the genomes of the different PCV2 isolates share higher sequence identities (≥94%) (Olvera et al., 2007). PCV2 strains isolated from Europe and North America around 1998, shared >96% of the genome, whereas the intra-continental sequence identity was even higher (>99%) (Meehan et al., 1998). Two major PCV2 genotypes have been identified in most countries experiencing cases of PMWS (Grau-Roma et al., 2008a; Olvera et al., 2007; Segalés et al., 2008) and recently a third genotype was detected in Denmark (Dupont et al., 2008). To unite the definitions and nomenclature of PCV2 genotypes between research groups, Segalés et al. (2008) suggested that the *cap* gene is used for genotyping of PCV2 strains, since phylogenetic trees based on ORF2 corresponds to trees based on the whole PCV2 genome. Furthermore, the following nomenclature for PCV2 genotypes has been suggested: PCV-2a, PCV-2b and PCV-2c (Opriessnig et al., 2007; Segalés et al., 2008). As the outcome of the PCV2 infection is quite inconsistent, there has been much debate of whether some PCV2 genotypes are more virulent than others. Early studies, did not find an association between PCV2 strains and severity of disease (Larochelle et al., 2003; Pogranichniy et al., 2002), but recently several authors have agreed upon that PCV-2b seems to be more virulent, based on studies of naturally occurring PCVDs. It has been shown that a shift in genotype from PCV-2a to -2b have occurred globally and since this was concurrent with the occurrence of PMWS, it was suggested that PCV-2b was a more adapted or virulent strain of PCV2 (Dupont et al., 2008). The most commonly isolated genotype associated with disease in Spain, is

PCV-2b and it was proposed that PCV-2a is less pathogenic (Grau-Roma et al., 2008a). In North America, both genotypes have been associated with outbreaks of PMWS, but there has been a shift from PCV-2a, as the most common genotype prior to 2005, to PCV-2b. Simultaneously with this, reports of increased severity of PCV2 associated lesions occurred (Carman et al., 2008; Desrosiers, 2007). The third genotype, PCV-2c has only been isolated from Denmark, from archived material collected prior to the emergence of PMWS (Dupont et al., 2008).

Other circoviruses

Several other viruses with a circular ssDNA genome have been identified in bacteria, plants, birds, man and other mammals. The bacteriophages are members of the *Inoviridae* or *Microviridae* and the plant-related viruses belong to the *Geminiviridae* or *Nanoviridae* (Day and Hendrix, 2005; Fane, 2005; Stanley et al., 2005; Vetten et al., 2005). The main part of the other viruses are contained in the *Circoviridae*, which is separated into two genera (genus *Circovirus* and *Gyrovirus*) according to the molecular biology of the viruses, and the remaining viruses belong to the new floating genus, *Anellovirus* (Biagini et al., 2005; Todd et al., 2005). Besides PCV1 and -2, the genus *Circovirus* includes beak and feather disease virus (BFDV), pigeon circovirus (PiCV), goose circovirus (GoCV) and canary circovirus (CaCV), which all have an ambisense genome of small size (Hino and Miyata, 2007; Todd et al., 2005). In recent years other circular viruses (duck circovirus, finch circovirus, gull circovirus) have been discovered in avian species and these are considered as tentative members of the genus *Circovirus* (Todd et al., 2005). The second genus, *Gyrovirus* only contains chicken anaemia virus (CAV), which has a negative sense genome (Todd, 2000). CAV is closely related to torque teno virus (TTV), a circular virus recently discovered in humans and which has been classified into the genus, *Anellovirus*. The smaller torque teno mini virus (TTMV) is also considered a member of this genus. TTV and TTMV have negative sense genomes and sequence motifs in common with CAV (Hino and Miyata, 2007; Todd et al., 2005). Viruses from the *Circoviridae* have narrow host ranges: PCV1 and -2 infect pigs, whereas the rest of the members infect avian species. Unlike this, TTV and TTMV can be isolated from human, non-human primates, pigs, ruminants, cats, dogs and chickens (Hino and Miyata, 2007; Todd et al., 2005).

In general, the circular viruses have many molecular biological similarities and the viral infections also have epidemiological, clinical and histopathological parallels. Some of these shared features

are the non-enveloped circular ssDNA genome and the replication process, which is similar to that of the porcine circoviruses (Biagini et al., 2005; Todd, 2000; Todd et al., 2005).

Pathogenesis of PCV2 infections

As the majority of sows have antibodies to PCV2, most piglets are protected by passively acquired immunity during the first weeks of life. Around 6-7 weeks of age the maternal antibody level has declined and simultaneously the piglets seroconvert, with maximal amounts of PCV2 antibodies produced from 12 weeks of age (Carasova et al., 2007; Grau-Roma et al., 2008b; Rodriguez-Arrioja et al., 2002). The age of infection varies among herds and between pigs, but mainly occurs in the interval from 4-11 weeks of age (Grau-Roma et al., 2008b) and seroconversion occurs around 10-21 days post infection (Fort et al., 2007; Meerts et al., 2006). PCV2 is spread vertically and horizontally, probably by direct oronasal, respiratory, urinary and/or fecal transmission, but how the virus enters the body, i.e. where and how the virus cross the body barriers, is not clear (Nauwynck et al., 2007; Opriessnig et al., 2007). Very little is known about the initial pathogenesis of the PCV2 infection in vivo, as only few experimental studies have focussed on the first hours (Stockhofe-Zurwieden et al., 2008) or the first week after infection (Fort et al., 2009; Yu et al., 2007). PCV2 DNA can be detected by polymerase chain reaction (PCR) in lungs, kidneys, liver and lymphoid organs as early as 3 days post inoculation (PI). At the same time replication can be observed in the lung, whereas replication in the other organs is not seen until 7 days PI (Yu et al., 2007). Viremia can be detected from 6-7 days PI, where PCV2 DNA is present in serum and/or in blood mononuclear cells (Fort et al., 2009; Pensaert et al., 2004; Yu et al., 2007).

PCV2 is taken up by macrophages and dendritic cells (DCs), which accumulate loads of virus without being affected by the infection, and these cells passively transport infective virus to different body compartments (Gilpin et al., 2003; Vincent et al., 2003). It has not been possible to find a specific cell type permissive of PCV2 replication, thus recent in vivo and in vitro studies have suggested that no such “specific cell type” exists, as PCV2 replication is detected in many cell types, e.g. epithelium (renal, intestinal, bronchiolar, hepatocytes, endothelium), fibrocytes, B-lymphocytes and macrophages (Pérez-Martín et al., 2007; Steiner et al., 2008; Yu et al., 2007). However, replication has not been detected in DCs (Steiner et al., 2008; Vincent et al., 2003).

In vitro studies of cells from the monocytic cell line, have shown that PCV2 quickly attaches to heparan sulphate and chrondroitin sulphate B glycosaminoglycans on the cellular membranes of cells, but the cellular internalization is slow and only occurs in half of the cells, to which PCV2 attaches (Misinzo et al., 2006; Misinzo et al., 2005) (figure 3). The virus enters monocytes/macrophages and DCs by clathrin-mediated endocytosis (Misinzo et al., 2005; Vincent et al., 2005) and the process probably also involves reorganization of the cellular actin cytoskeleton. Inside the cell, the pH of the clathrin-coated vesicles declines, which seems necessary for the release of virus into the cytoplasm (Misinzo et al., 2005). Epithelial cells are somewhat different, in these cells PCV2 can be internalized by the same mechanism, however, for efficient replication to occur, the virus has to enter by another pathway, i.e. a cholesterol- and dynamin-independent, but actin- and small GTPase-dependent pathway (Misinzo et al., 2009). In the cytoplasm of PK-15 cells

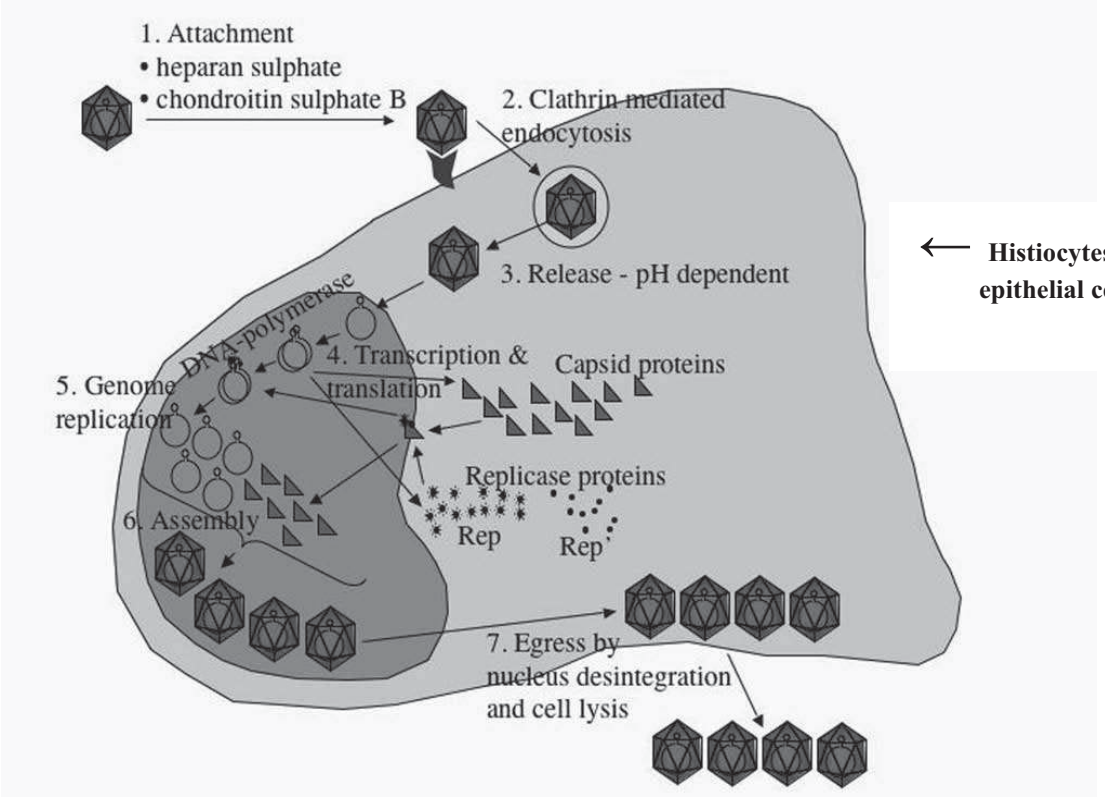

Fig. 3. Porcine circovirus type 2 (PCV2) infection and intracellular replication cycle. (1) PCV2 attaches to heparan sulphate and chrondroitin sulphate B glycosaminoglycans on the cellular surface. (2) Internalization occurs by clathrin-mediated endocytosis or by a cholesterol- and dynamin-independent, but actin- and small GTPase-dependent pathway. (3) The pH of the clathrin-coated vesicles declines and PCV2 is released into the cytoplasm. (4) to (6) Replication and production of viral particles takes from 24-36 hours. (7) Virus progeny is released from the cell by cell lysis. (Figure modified after Nauwynck et al., 2007)

viral capsid protein can be detected from 6-12 hours PI, and in the nucleus, capsid and replicase proteins are present 12-24 hours PI. Replication and production of viral particles takes from 24-36 hours and the virus progeny is released by cell lysis, which coincides with nuclear disintegration (Cheung and Bolin, 2002; Meerts et al., 2005a). However, in in vitro studies of porcine alveolar macrophages and foetal myocardiocytes, no virus progeny was detected, although viral antigens were present in the nucleus 48 hours PI (Meerts et al., 2005a).

Lymphoid depletion of the lymphoid organs is a characteristic lesion of PMWS. Experimental infection studies (Yu et al., 2007) and natural cases of PMWS (Sarli et al., 2001; Segalés et al., 2004a) have shown that early in the infection, depletion is manifested by loss of lymphoid follicles (mainly B-cell dependent areas), which becomes more severe at later stages of disease. However the mechanism responsible for the depletion is still unidentified (Opriessnig et al., 2007; Ramamoorthy and Meng, 2008). Reduced proliferation of lymphoid cells (Mandrioli et al., 2004; Resendes et al., 2004) and replication-associated viral-induced damage (Lin et al., 2008) have been suggested to be responsible for the lymphocyte depletion. Also, apoptosis of lymphocytes have been observed by some authors in vivo (Shibahara et al., 2000) or in vitro (Chang et al., 2007; Liu et al., 2005), however others have not been able to confirm these findings (Mandrioli et al., 2004; Resendes et al., 2004). Recently, it was suggested that ORF3 is important for the development of lymphoid depletion, and the effect is believed to be apoptosis-related, as in vitro studies have shown that the ORF3 protein triggers apoptosis in PCV2 infected cells (Liu et al., 2005). In vivo studies of mice (Liu et al., 2006) and piglets (Karuppannan et al., 2009), experimentally inoculated with wild-type PCV2 or an ORF3 deficient mutant strain of PCV2, showed that the pathogenicity of the mutant strain was attenuated, and that the infection was not associated with lymphocyte depletion and histiocytic infiltration, as seen with the wild-type virus. Thus it is proposed that ORF3 plays an important role in the viral pathogenesis (Karuppannan et al., 2009; Liu et al., 2006).

Porcine circovirus diseases

PMWS was first described in Canada in 1996 by Harding and Clark (1997), since then PCV2 has been associated with several disease complexes, which collectively have been named porcine circovirus diseases (PCVDs) (Allan and McNeilly, 2006). Several reports of PCVDs exist, but until now it has only been possible to experimentally reproduce PMWS and PCV2 associated

reproductive failure. Thus, the tentative PCVDs are: porcine respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS), proliferative and necrotizing pneumonia (PNP), congenital tremor (CT), PCV2 associated enteritis, necrotizing lymphadenitis and exudative epidermitis (Grau-Roma and Segalés, 2007; Jensen et al., 2006; Kim and Chae, 2004b; Kim and Chae, 2005; Opriessnig et al., 2007; Stevenson et al., 2001). Besides causing clinical manifest disease, PCV2 is able to cause subclinical infection, where the propagation to clinical disease depends on several factors associated with the host, viral strain and environment (Opriessnig et al., 2007) (figure 4). In general, to diagnose PCVD, the mere identification of PCV2 is not regarded as diagnostic. This depends on the fact that PCV2 occurs ubiquitous and can be detected in low amounts in tissues of subclinically infected and convalescent pigs (Segalés et al., 2004b). Thus, since PCR is a very sensitive method, able to detect small amounts of viral DNA, it is not regarded as a diagnostic method of PCVD. Instead immunohistochemistry (IHC) or in situ hybridization (ISH) should be used to detect PCV2 within lesions (Chae, 2004; Harding and Halbur, 2002; Opriessnig et al., 2007).

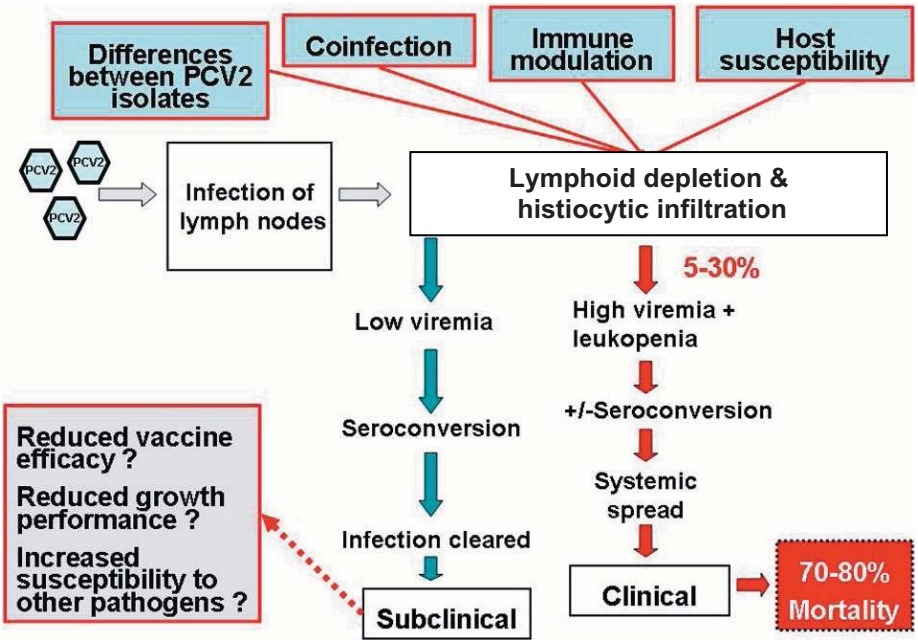

Fig. 4. Schematic presentation of factors believed to influence the outcome of porcine circovirus type 2 infections, i.e. subclinical infection or clinical manifest porcine circovirus disease. (Figure modified after Opriessnig et al., 2007)

Postweaning multisystemic wasting syndrome

Clinical signs

PMWS mainly affects pigs of 7-15 weeks of age (Darwich et al., 2004), although in recent years there have been several reports on PMWS in older pigs (D'Allaire et al., 2007; Carman et al., 2008; Fachinger et al., 2008). The disease prevalence varies from herd to herd and between countries. On affected farms the morbidity is about 4-30% and 70-80% of these pigs will die (Segalés and Domingo, 2002). Clinical manifestations are non-specific, but the major findings are increased mortality, wasting, dyspnoea, pallor of the skin, diarrhoea and, less frequently, jaundice (Segalés et al., 2005).

Lesions

Gross lesions include pulmonary lesions, i.e. non-collapsed lungs and/or cranioventral consolidation, local or generalised lymph node enlargement and thymus atrophy. Further findings can be liver atrophy, “white spotted kidneys”, gastric ulceration and serous fat atrophy (Harding and Clark, 1997; Ladekjaer-Mikkelsen et al., 2002; Segalés et al., 2004b). Histopathologic findings are primarily associated to the lymphoid organs, but lesions, mainly lymphohistiocytic infiltrations, can also be detected in a vide variety of other organs. Characteristic lesions are detected in the lymph nodes, where early signs are histiocytic infiltration in the subcapsular sinus system and lymphocyte depletion, mainly affecting the B-lymphocytes in the lymphoid follicles. Furthermore, multinucleate giant cells can be present in the lymphoid follicles, the sinus system and the parafollicular areas. In later stages, the parafollicular areas also become affected by depletion of B- and T-lymphocytes and an increase in the number of histiocytes are found. Finally, only stromal tissue, histiocytes, multinucleate giant cells and few lymphocytes are left in the lymph nodes (Sarli et al., 2001; Segalés et al., 2005). Occasionally necrotizing lymphadenitis is detected (Segalés et al., 2004b). Grape-like basophilic inclusion bodies can be observed in the cytoplasm of histiocytes, and occasionally, in the pulmonary and renal epithelial cells (Huang et al., 2008). Similar lesions can be detected in other secondary lymphoid tissues, i.e. spleen, peyer´s patches, tonsils and occasionally in the bronchus-associated lymphoid tissue (BALT) (Segalés et al., 2004b). In thymus, the lesions are similar to those described for the lymph nodes and depletion first affects the cortical lymphocytes (Segalés et al., 2005). The main finding in the lungs is interstitial pneumonia as evidenced by mononuclear infiltration in alveoli, and in the peribronchial- and bronchiolar connective tissues; suppurative bronchopneumonia and occasionally necrotizing pneumonia can

also be observed (Quintana et al., 2001; Segalés et al., 2005). The kidneys are affected by variants of interstitial nephritis, i.e. the inflammatory infiltrate can be granulomatous, lymphoplasmacytic and/or lymphohistiocytic (Sarli et al., 2008). Furthermore, non-suppurative vasculitis is often detected (Harding and Clark, 1997). The main observation in the gastrointestinal tract is submucosal oedema and infiltration of lymphohistiocytic cells in the mucosa (Harding and Clark, 1997). In the liver lymphohistiocytic interstitial hepatitis, single cell necrosis and perilobular fibrosis, can be seen (Quintana et al., 2001; Rosell et al., 2000a). Other organs may also contain foci of lymphohistiocytic infiltration.

Diagnosis

Based on the variable and unspecific findings, it is somewhat difficult to diagnose PMWS in herds, thus a herd definition of PMWS has been proposed by the EU-financed project "Control of porcine circovirus diseases (PCVDs): Towards improved food quality and safety (PCVD 513928)" (Bækbo, 2005). To make a herd diagnosis of PMWS, there should be an excessive increase in mortality during the postweaning period, compared to the historical data of the herd or the national level. Furthermore, at least 1 out of 5 examined pigs should have clinical signs consistent with PMWS (as described above) and characteristic histopathological lesions in the lymphoid tissues (lymphocyte depletion and histiocytic infiltration, in addition multinucleate giant cells and/or inclusion bodies can be present). Within these lesions moderate or massive amounts of PCV2 should be detected by IHC or ISH, otherwise the condition is not regarded as PMWS. Recently, Opriessnig et al. (2007) have defined PMWS as a systemic infection, where at least 2 tissues should be affected (e.g. 2 lymph nodes, lung and lymph nodes or lung and liver), versus local disease, where only 1 specific organ system is affected (e.g. lungs, reproductive organs or intestines).

PCV2 associated reproductive failure

Clinical signs

Although, PCV2 is not believed to be a major cause of reproductive problems (Bogdan et al., 2001; Maldonado et al., 2005), several case reports exist on PCV2 associated reproductive failure, mainly from newly established herds with first parity gilts (Brunborg et al., 2007; Josephson and Charbonneau, 2001; Ladekjaer-Mikkelsen et al., 2001; O'Connor et al., 2001; Pittman, 2008; West et al., 1999). The clinical findings accompanying PCV2 associated reproductive failure, are an increase in the number of abortions, stillborns, mummies and weak born piglets, affecting naïve

sows/gilts (Brunborg et al., 2007; Johnson et al., 2002; Pittman, 2008). Except for anorexia in a few animals (Park et al., 2005), no symptoms have been described in the affected sows (Brunborg et al., 2007; West et al., 1999). The reproductive disorders are reported to resolve spontaneously, typically after 6-8 weeks, with the sows returning to normal reproductive performances (Brunborg et al., 2007; O'Connor et al., 2001; Pittman, 2008).

Lesions

Gross lesions in the foetuses are: enlarged lymph nodes, hydrothorax, ascites, and dilatation and discoloration of the heart (Brunborg et al., 2007; Johnson et al., 2002; Mikami et al., 2005). The main histopathological findings in the stillborn and mummified foetuses are multifocal non-suppurative and/or fibrotic and/or necrotic myocarditis, sometimes with mineralization. In the live born piglets additional findings are: necrosis and/or lymphocyte depletion in lymphoid organs, necrotic foci in the liver, and pneumonia, mainly interstitial (Brunborg et al., 2007; Mikami et al., 2005; Pittman, 2008).

Diagnosis

The diagnosis of PCV2 associated reproductive failure is based on the increased number of abortions, stillborn and/or mummified foetuses, in combination with the detection of PCV2 in the myocardial lesions (Segalés et al., 2006).

Porcine respiratory disease complex

Aetiology and clinical signs

PRDC, also referred to as “the 18-week wall” is a multi-etiologic pneumonia, affecting pigs around 14 to 22 weeks of age (Kim et al., 2003; Thacker, 2001). Several viruses, bacteria and mycoplasmas are involved in this disease complex and the pathogens are isolated in varying frequencies according to herd and national health status. However, some agreement exist about the following pathogens being most commonly isolated: porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), PCV2, *Pasteurella multocida* and *Mycoplasma hyopneumoniae* (Chiou et al., 2004; Choi et al., 2003; Harms et al., 2002; Thacker, 2001). But other pathogens have also been associated with PRDC, e.g. pseudorabies virus, porcine respiratory coronavirus, porcine parvovirus (PPV), *Streptococcus suis*, *Actinobacillus pleuropneumoniae* and [*Haemophilus*] *parasuis* (Halbur, 1998; Kim et al., 2003; Thacker, 2001). The course of disease is

mainly insidious, ending in chronicity, but acute onset has been associated with PRDC (Harms et al., 2002). Morbidity varies from 30-70% and mortality is 2-10%, but can be as high as 20% (Halbur, 1998; Harding and Halbur, 2002; Harms et al., 2002). Clinical manifestations are dyspnoea, coughing, fever, lethargy, anorexia, ill thrift and reduced growth (Chae, 2005; Kim et al., 2003; Thacker, 2001).

Lesions

Lesions are mainly related to the cranioventral parts of the lungs. The lungs are mottled and the colour varies from tan-to-purple, furthermore the lung tissue is consolidated and fails to collapse (Harms et al., 2002). Histopathology varies according to the involved pathogens. Findings that imply involvement of PCV2 are lymphohistiocytic interstitial pneumonia with bronchiolitis and bronchiolar fibrosis (Opriessnig et al., 2007). In cases where PCV2 and PRRSV, SIV or *M. hyopneumoniae* are detected as coinfections, the following lesions can be seen: suppurative bronchopneumonia, hyperplasia of type II pneumocytes, epithelial necrosis, BALT hyperplasia and fibrinosuppurative pleuritis (Harms et al., 2002).

Diagnosis

As this disease complex is multifactorial, the involved pathogens and histopathologic findings will depend on the specific case examined. However, the lung lesions should involve the cranioventral part of the lungs and several different pathogens should be detected. To diagnose PCV2 associated PRDC as opposed to PMWS, the following criteria should be met: only the lungs should be affected, PCV2 should be present in the lung lesions and most importantly, no lesions characteristic of PMWS should be present in the lymphoid organs (Chae, 2005; Opriessnig et al., 2007).

Porcine dermatitis and nephropathy syndrome

Aetiology and clinical signs

PDNS was first recognized in 1991/1992 in the United Kingdom (Smith et al., 1993). Since then several have reported similar findings and PDNS is now expected to occur worldwide (Phaneuf et al., 2007; Segalés et al., 1998; Thibault et al., 1998; Wellenberg et al., 2004). The pathogenetic mechanism of PDNS is believed to be a type III hypersensitivity reaction, with deposition of immune complexes in the vascular walls (Smith et al., 1993; Wellenberg et al., 2004). The exact aetiology is not known, but PRRSV and/or PCV2 are the pathogens detected most frequently in

affected animals (Choi and Chae, 2001; Majzoub et al., 2005; Phaneuf et al., 2007; Ritzmann et al., 2005; Rosell et al., 2000b; Segalés et al., 1998; Thibault et al., 1998; Wellenberg et al., 2004), however *P. multocida* and streptococcus spp. have also been linked with the disease (Drolet et al., 1999; Lainson et al., 2002). The disease prevalence is low (about 1%) (Drolet et al., 1999; Smith et al., 1993) and usually pigs from 6 to 14 weeks of age are affected (Phaneuf et al., 2007; Thibault et al., 1998). The condition is fatal in severely affected pigs, whereas mild lesions may resolve spontaneously (Drolet et al., 1999; Smith et al., 1993). The most characteristic findings are multifocal to coalescing, slightly swollen and red skin lesions, mainly on the hind limbs and caudal parts of the body. If the pig survives, the lesions become covered by brown crusts. Furthermore the pigs may be depressed, and have fever, subcutaneous oedema and a stiff gait (Drolet et al., 1999; Phaneuf et al., 2007; Thibault et al., 1998).

Lesions

Besides the skin lesions, other gross lesions are: swollen pale kidneys with petechia, subcutaneous oedema and mild consolidation of the lung tissue (Phaneuf et al., 2007; Smith et al., 1993; Thibault et al., 1998). The histopathology is characterised by systemic necrotizing vasculitis of small and medium sized arterioles, mainly affecting the skin, kidneys and spleen (Drolet et al., 1999; Phaneuf et al., 2007). Lesions in the skin compare to haemorrhagic epidermitis (Smith et al., 1993; Thibault et al., 1998), and in the kidneys glomerulonephritis and occasionally interstitial nephritis are revealed (Phaneuf et al., 2007; Wellenberg et al., 2004). In the lymph nodes lymphocyte depletion of the lymphoid follicles and presence of multinucleate giant cells might be observed (Chae, 2005; Wellenberg et al., 2004).

Diagnosis

The diagnosis of PDNS depends on the presence of typical gross and histological lesions, only in a few animals. Furthermore, other diseases with similar lesions should be excluded (Segalés, 2002).

Proliferative and necrotizing pneumonia

Aetiology and clinical signs

PNP was first recognized in Canada in the late 1980s as a severe respiratory disease, which occurred sporadically and mainly affected pigs of 4-16 weeks of age (Morin et al., 1990; Thomson and Carman, 1991). Afterwards, PNP has been described in other countries, based on retrospective

studies (Grau-Roma and Segalés, 2007; Pesch et al., 2000). Most agree upon that PCV2, PRRSV and possible also SIV type A are associated with PNP, but the suggested significance of each of these agents differ between the surveys (Drolet et al., 2003; Grau-Roma and Segalés, 2007; Larochelle et al., 1994; Pesch et al., 2000). Affected pigs develop dyspnoea and fever, and in pigs that survive, wasting and growth retardation can be observed (Morin et al., 1990).

Lesions

The lungs appear dry, meaty and do not collapse, furthermore the lung tissue of the cranial- and middle lung lobes, and the caudal parts of the caudal lung lobes are consolidated (Morin et al., 1990). Histological findings are fibrin exudation, hyaline membrane formation and conglomerates of necrotic cell debris in the alveolar lumen, and extensive hyperplasia of type II pneumocytes. Furthermore, necrotizing bronchiolitis, lymphohistiocytic interstitial pneumonia and/or suppurative bronchopneumonia can be seen in some pigs (Drolet et al., 2003; Grau-Roma and Segalés, 2007; Morin et al., 1990).

Diagnosis

When diagnosing PNP, the lesions should involve most of or the entire lung and the following histopathological findings should be revealed: deposits of fibrin and accumulations of necrotic inflammatory cells in the alveolar lumen, together with extensive proliferation of the alveolar and bronchiolar epithelium (Morin et al., 1990).

Other PCVDs

CT is associated with myelin deficiency, which causes muscle tremors in neonatal swine. The disease usually resolves spontaneously, when the piglets are around 4 weeks of age (Stevenson et al., 2001). Several types of CT are known, and the pathogenesis of CT type A2 is believed to be associated with a viral infection, but the exact agent has not been identified (Ha et al., 2005). Some have detected PCV2 in the central nervous system of piglets affected by CT type A2 (Choi et al., 2002; Stevenson et al., 2001), whereas others have not (Brunborg et al., 2007; Ha et al., 2005; Kennedy et al., 2003). However in one of these studies (Brunborg et al., 2007), CT was observed in relation to a case of PCV2 associated reproductive failure. Thus, the role of PCV2 in CT is still controversial.

In growing and finishing pigs PCV2 has also been detected within lesions, in cases of granulomatous enteritis (Jensen et al., 2006; Kim et al., 2004; Kolb et al., 2006) and necrotizing lymphadenitis (Kim and Chae, 2005). Furthermore, it has been suggested that the PCV2 infection can be a predisposing factor in the development of exudative dermatitis in piglets (Kim and Chae, 2004b; Wattrang et al., 2002).

Experimental studies

Reproduction of PMWS

Several have tried to reproduce PMWS in experimental settings, but with varying success. Instead, the inoculation studies often have induced subclinical PCV2 infection without clinical signs of PMWS and with low to moderate amounts of virus in the tissues. PMWS is characterized as a multifactorial disease entity and many different risk factors/triggers have been identified as important for the expression of the disease. Hence, experimental models based on the inoculation of PCV2 alone have only induced PMWS in some settings and this mainly in a low fraction of animals (Albina et al., 2001; Bolin et al., 2001; Harms et al., 2001; Hirai et al., 2003; Ladekjaer-Mikkelsen et al., 2002; Magar et al., 2000; Okuda et al., 2003), whereas other similar models failed to reproduce PMWS (Darwich et al., 2008; Ellis et al., 1999; Fenaux et al., 2002; Meerts et al., 2005b; Opriessnig et al., 2008; Pogranichnyy et al., 2000; Sanchez et al., 2004).

Based on the incidental finding of coinfection with PPV in a PCV2 inoculation study, it was suggested that both viruses were responsible for the expression of PMWS (Ellis et al., 1999). Later on, others tried to induce PMWS by dual inoculations with PCV2 and PPV (Allan et al., 1999; Hasslung et al., 2005; Kim and Chae, 2004a; Krakowka et al., 2000) or PRRSV (Harms et al., 2001; Rovira et al., 2002) or *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004) or porcine TTV (Ellis et al., 2008). The success rate of these studies has been quite high, though not all dual infections with PPV or PRRSV have caused PMWS (Allan et al., 2000a; Allan et al., 2000b; Ostanello et al., 2005). Based on the results obtained by the coinfections, Krakowka et al. (2001) tested the hypothesis that besides the PCV2 infection, immunostimulation was the mechanism, which induced PMWS. This was tested by injecting PCV2-inoculated gnotobiotic piglets with keyhole limpet hemocyanin emulsified in incomplete Freund´s adjuvant (KLH/ICFA), which proved to be very

successful in developing PMWS. Though, later studies of PCV2 inoculation and concurrent KLH/ICFA injections in specific pathogen free (SPF) piglets showed a lower rate of success (Grasland et al., 2005; Ladekjaer-Mikkelsen et al., 2002). Other ways of trying to achieve immunostimulation have been by concurrent vaccination (Allan et al., 2007; Krakowka et al., 2007; Opriessnig et al., 2006b; Opriessnig et al., 2003) or by injection of lipopolysaccharide (Fernandes et al., 2007). Among the above studies, PMWS was only induced in 1 study by the use of vaccines that contained mineral oil-adjuvant, thus this was believed to be the triggering factor (Krakowka et al., 2007). Furthermore, the effect of PCV2 inoculation and concurrent immunosuppression has been tested in gnotobiotic piglets by the use of cyclosporine or steroid treatments, which only reproduced PMWS in a low fraction of the piglets in some studies (Krakowka et al., 2002), whereas other studies did not reproduce PMWS (Krakowka et al., 2002; Meerts et al., 2005b). Others have shown that the immune status of the sow and her offspring might be important in the expression of PMWS. Recently, PMWS was reproduced in piglets by PCV2 inoculation of sows at insemination or during gestation, combined with deprivation of passive immunity of the offspring (i.e. deprivation of colostrum) or by postnatal immunostimulation (i.e. injection of KLH/ICFA) or by postnatal inoculation with PPV (Ha et al., 2008; Rose et al., 2007).

So far, mainly PCV-2a has been used for inoculation of pigs in experimental settings, and in the few studies where the virulence of PCV-2a and PCV-2b has been compared, no difference was observed between the genotypes in the ability to reproduce PMWS (Fort et al., 2008; Gauger et al., 2008; Lager et al., 2007; Opriessnig et al., 2008). Other variables in the experimental settings are the age, breed and origin of the pigs used. Like this, gnotobiotic- (Ellis et al., 2008; Krakowka et al., 2001; Krakowka et al., 2000), caesarean-derived/colostrum-deprived- (Bolin et al., 2001; Harms et al., 2001; Hasslung et al., 2005; Okuda et al., 2003), conventional- (Albina et al., 2001; Opriessnig et al., 2004; Rovira et al., 2002), colostrum-deprived conventional- (Allan et al., 1999; Hasslung et al., 2005; Hirai et al., 2003; Kim and Chae, 2004a) and SPF piglets (Albina et al., 2001; Ladekjaer-Mikkelsen et al., 2002; Magar et al., 2000) have been used in studies, where PMWS was reproduced. Furthermore, experimental inoculation studies have shown that the susceptibility to PCV2 associated disease and lesions, might be increased in Landrace pigs compared to other breeds (Opriessnig et al., 2006a; Opriessnig et al., 2009).

The effect of all these different variables on the induction of PMWS has been characterized in a meta-analysis based on 44 experimental settings (Tomás et al., 2008). Here it was found that the most successful way to reproduce PMWS, would be to include colostrum-deprived piglets younger than 3 weeks of age, inoculate with high doses of PCV2 (>10^5 50% tissue culture infectious dose per pig) from genotype b, together with another porcine pathogen. But despite all the different experimental trials to date, still no robust model for the reproduction of PMWS exists.

Reproduction of PCV2 associated reproductive failure

In contrast to the experimental PMWS studies, PCV2 associated reproductive failure can be induced by PCV2 inoculation alone, with quite successful results (Johnson et al., 2002; Rose et al., 2007; Sanchez et al., 2001). Studies of porcine embryos have shown that PCV2 can infect embryonic cells of hatched blastocysts and induce embryonic death (Mateusen et al., 2007; Mateusen et al., 2004). Reproductive failure has been induced by inoculation of PCV2 directly into foetuses at different stages of gestation, which has been carried out by transuterine injection during laparotomy (Johnson et al., 2002; Pensaert et al., 2004; Sanchez et al., 2003; Sanchez et al., 2001) or by ultrasound needle-guided transabdominal injection (Yoon et al., 2004). In these studies, it was shown that foetuses inoculated before immunoincompetence (<70 days of gestation) were most severely affected by the infection, i.e. developed lesions, had high viral loads mainly in the hearts and died, compared to foetuses inoculated after immunocompetence. The latter mounted antibodies against PCV2 and the virus was detected at lower levels in different tissues. Furthermore, histological lesions and deaths were only detected in few of these foetuses (Johnson et al., 2002; Pensaert et al., 2004; Sanchez et al., 2001). PCV2 can be transmitted from infected immunoincompetent foetuses to neighbouring un-inoculated foetuses, however this has only been shown in one study (Pensaert et al., 2004). Reproductive failure has also been reproduced by vertical transmission of PCV2. Naïve sows that have been oronasally inoculated with PCV2 late in the gestation, have transferred the virus to their foetuses (Park et al., 2005). It has also been possible to transfer PCV2 to foetuses of antibody positive sows, which were inoculated with PCV2 oronasally during pregnancy (Nielsen et al., 2004) or by intrauterine inoculation at insemination (Rose et al., 2007). Like in the PMWS experimental settings, most have performed the PCV2 inoculations with genotype a (Mateusen et al., 2004; Pensaert et al., 2004; Sanchez et al., 2001), and recently, it was shown that there is no apparent difference in disease expression between immunoincompetent foetuses inoculated with PCV-2a or -2b (Lefebvre et al., 2009).

Chapter 3 Manuscripts I-IV

Manuscript I

Detection of porcine circovirus type 2 and viral replication in primary lymphoid organs from naturally and experimentally infected pigs

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Detection of porcine circovirus type 2 and viral replication in primary lymphoid organs from naturally and experimentally infected pigs

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Abstract

Several circular single-stranded (ss) DNA viruses, including porcine circovirus type 2 (PCV2) have been identified in birds and mammals. These ssDNA viruses and the related diseases, share various molecular biological, epidemiological, clinical and histopathological features. This includes the replication process, where an intermediate, circular double-stranded DNA form (replicative form, RF) is generated. Furthermore, most of these viruses infect and/or replicate specifically in the primary lymphoid organs. The aim of this study was to asses the role of the primary lymphoid organs in the pathogenesis of the PCV2 infection and postweaning multisystemic wasting syndrome (PMWS) in swine. This was carried out by histopathological examination of the thymus and bone marrow from pigs experimentally inoculated with PCV2, defined as subclinically infected animals, or naturally PMWS-affected pigs. Furthermore, in situ hybridization (ISH) for presence of PCV2 nucleic acids and PCV2 replication was performed. For this, two ISH probes were used; the complementary probe (CP) detected PCV2 irrespective of replicative status, while the replicative form probe (RFP) detected the RF of PCV2, which is only present during viral replication. PCV2 was not detected in the thymus or bone marrow from the experimentally PCV2-inoculated pigs or any of the control animals. In the PMWS-affected pigs, 95% of the thymuses were positive for PCV2 by CP ISH and 37% of these thymuses also supported viral replication. By CP ISH, PCV2 was detected in 48% of the bone marrows and 31% of these bone marrows also had evidence of

viral replication. The proportion of cells labelled by the RFP was relatively lower than cells labelled by the CP. In both organs, the cell type primarily labelled by the CP and RFP was identified as histiocyte by morphological criteria. In thymus, there were few lymphocyte-like cells that were positive for PCV2 nucleic acids and virus replication. In conclusion, PCV2 nucleic acids and PCV2 replication were indeed found in both bone marrow and thymus of PMWS-affected pigs, but there was no evidence that PCV2 replication is supported by cell types specific for thymus or bone marrow in cases of PMWS.

Keywords: Porcine circovirus type 2 (PCV2); Postweaning multisystemic wasting syndrome (PMWS); Bone marrow; Thymus; Swine

Introduction

Porcine circovirus type 2 (PCV2), a circular single-stranded (ss) DNA virus, is recognized as the causative agent of postweaning multisystemic wasting syndrome (PMWS) (Chae, 2004; Ghebremariam and Gruys, 2005). PMWS is clinically characterized by increased mortality in pigs of 7-15 weeks of age, wasting, dyspnoea and, less frequently, by diarrhoea and jaundice (Darwich et al., 2004; Ghebremariam and Gruys, 2005). In the last decades several circular ssDNA viruses, including PCV2, have been detected in birds, man and other mammals. These viruses belong to the family *Circoviridae* (genus *Circovirus* and *Gyrovirus*) or *Anellovirus* (floating genus). These viruses have many molecular biological similarities and the viral infections also have some epidemiological, clinical and histopathological parallels. Shared features of the viruses are the non-enveloped circular ssDNA genome and the replication process, where the virus goes through an intermediary double stranded (ds) form, the replicative form (RF). The generation of the RF involves de-novo DNA synthesis, a process depending on the mitosis and nuclear enzymes of the host cell (Todd, 2000; Biagini et al., 2005; Todd et al., 2005). PCV2, beak and feather disease virus (BFDV), pigeon circovirus (PiCV), goose circovirus (GoCV) and chicken anaemia virus (CAV) are widely spread and able to cause both clinical and subclinical infections, whereas porcine circovirus type 1, Torque teno virus (TTV) and Torque teno mini virus only have been associated with subclinical disease (Todd, 2000; Smyth et al., 2005; Marlier and Vindevogel, 2006; Opriessnig et al., 2007; Hino and Miyata, 2007). Most of these viruses (CAV, BFDV, PiCV, GoCV, PCV2) affect lymphoid tissues and cause several lesions like atrophy, necrosis and lymphocyte depletion, leading to immunosuppression and secondary infections (Todd, 2000; Smyth et al., 2005). Both primary

(thymus, bone marrow, bursa of Fabricius) and secondary lymphoid tissues (lymph nodes, spleen, tonsil, bronchial-associated lymphoid tissue, gut-associated lymphoid tissue) may be affected, but the predilection site depends on the specific virus (Todd, 2000; Hino and Miyata, 2007). In most cases the viruses can also be isolated from other organs and tissues such as lung, liver, blood, kidney, brain, heart, intestine, trachea, skin and for avian species, from crop, claws, beak and feathers (Todd, 2000; Marlier and Vindevogel, 2006; Hino and Miyata, 2007).

An important step in understanding the pathogenesis of viral diseases is the identification of cells permissive of replication. The replication process of the circular viruses is complex and for most of these viruses, the cells supporting replication are still to be disclosed. It has been shown that CAV replicates in bone marrow (haematocytoblasts), thymus (lymphocyte precursors), spleen and gastrointestinal-tract (Todd, 2000), and that TTV replicates in bone marrow (erythroid series and megacaryocytes), peripheral blood mononuclear cells and liver (Hino and Miyata, 2007; Kakkola et al., 2007). Much effort has been put into finding cells permissive of PCV2 replication in vivo, but only recently relevant methods have become available. Three different approaches for detecting replication in vivo have been described; Yu et al. (2007) measured the level of spliced PCV2 capsid messenger RNA (mRNA) by polymerase chain reaction (PCR) in tissue homogenates, Hamberg et al. (2007) combined in situ hybridization (ISH) for PCV2 and immunohistochemistry (IHC) for ssDNA and dsDNA, whereas Pérez-Martín et al. (2007) used an ISH probe that hybridized exclusively with the RF of the virus. A common feature for these studies was that relatively few thymus samples were included and, in regard of bone marrow, only one study investigated a small number of samples. Since PCV2 is closely related to other circular viruses, which often affect and/or replicate in the primary lymphoid organs, there have been speculations on which role the primary lymphoid organs play in the PCV2 infection (Darwich et al., 2004; Opriessnig et al., 2007).

The present study was conducted to assess the role of the primary lymphoid organs during the PCV2 infection in swine. Thymus and bone marrow from subclinically infected pigs that were experimentally inoculated with PCV2, and naturally PMWS-affected pigs were examined by histopathology and ISH for the presence of PCV2 nucleic acids and PCV2 replication.

Materials and Methods

Subclinically infected pigs (experimental infection study)

In an experimental infection study (Fernandes et al., 2007; Fort et al., 2009), 36 caesarean-derived, colostrum-deprived (CD/CD) Landrace piglets were obtained from a Spanish farm seropositive for PCV2. At seven days of age, 24 animals were intranasally and orally inoculated with $10^{5.2}$ 50% tissue culture infectious dose per millilitre ($TCID_{50}$ ml^{-1}) of a Spanish PCV2 strain (Burgos), whereas 12 control animals were mock inoculated with a sterile cell culture medium. Respectively, at 5, 8 and 29 days post inoculation (DPI), 6 PCV2-inoculated and 2 control animals, 7-PCV2 inoculated and 2 control animals and 11 PCV2-inoculated and 8 control animals, were euthanized and tissues, including thymus and bone marrow, were sampled. Thymus was not obtainable in one control pig euthanized at 29 DPI. The PCV2-inoculated piglets seroconverted between 14-21 DPI, all control animals remained seronegative. No PCV2 infection-related clinical signs or lesions (Sorden, 2000; Segalés, 2002) were detected in the control animals. In the PCV2-inoculated group, slight lymphoid depletion, occasional granulomatous inflammation and presence of multinucleated giant cells were observed in the secondary lymphoid organs at 8 DPI (n = 2) and 29 DPI (n = 10). Nevertheless, no PCV2 infection-related clinical signs or lesions were observed in other PCV2-inoculated animals. PCV2 was not detected by ISH in any tissues from the control animals, whereas 1/7 and 8/11 of the PCV2-inoculated piglets had low amounts of PCV2 in a few lymph nodes at 8 and 29 DPI, respectively.

Naturally PMWS-affected pigs (field study)

From a case-control study in PMWS-affected farms (Grau-Roma et al., 2008), 33 animals with PMWS and 29 age-matched, healthy control animals were selected for the present study. The animals had been euthanized at 12-20 weeks of age and tissues, including thymus and bone marrow were harvested. However, among the selected animals thymus could not be identified in 13 of the PMWS-affected animals and in 1 healthy animal. The PMWS diagnosis was based on the clinical signs, presence of characteristic histopathologic findings of lymphoid depletion and granulomatous inflammation and detection of moderate to massive PCV2 load in lymphoid tissues, all in accordance with accepted standards (Sorden, 2000; Segalés, 2002).

Histopathology

Within 45 min after euthanization, tissue samples of thymus and bone marrow were fixed by immersion in 10% neutral buffered formalin for 24 h. After fixation, the bone marrow samples were decalcified in Decalcifier II (Surgipath Europe Ltd., United Kingdom) for 1-2 h. Next, all tissue samples were dehydrated, embedded in paraffin wax, cut in 3-5 µm thick sections and mounted on conventional glass slides, SuperFrost®Plus slides (Mensel-gläser, Germany) or silanised Chemmate capillary gap microscopic slides (DAKO, Denmark) for histochemistry, IHC or ISH, respectively. All tissue sections were stained with hematoxylin and eosin (HE) according to standard procedures. Selected sections of bone marrow and thymus representing the observed histopathological variation, were further examined by different histochemical and immunohistochemical staining procedures. All staining procedures applied are listed in table 1.

In the bone marrow, the following was examined by semiquantitative methods as suggested by the Society of Toxicologic Pathology (STP) Immunotoxicology Working Group (2005); general cellularity, myeloid to erythroid (M:E) ratio, and quantity and morphology of megakaryocytes, erythroid cells, granulocytic cells and other cells (including lymphocytes, plasma cells, monocytes, histiocytes, osteoclasts, osteoblasts and reticular stromal cells) (Kohler, 1956; Thorn, 2000; Harvey, 2001). The general marrow cellularity was presented in grades of hypocellularity as: normocellular (0), slightly hypocellular (+), hypocellular (++) or markedly hypocellular/acellular (+++) (figure 1). The M:E ratios were divided into six categories and for each group of animals a mean M:E ratio and standard deviation (SD) was calculated (table 2). The examination also included a description of other lesions, when present. The finding of an eosinophilic homogenous amorphous material surrounding the adipocytes was observed in some of the bone marrows stained by HE. This material is from hereon referred to as “fat-associated eosinophilic material” (FAEM). The amount of FAEM was scored semiquantitatively as: absent (0), low (+), moderate (++) or high (+++) (figure 2 a, c).

Thymus was evaluated semiquantitatively as suggested by Elmore (2006) and the STP Immunotoxicology Working Group (2005). Thus, the quantity and morphology of lymphocytes in the cortex and medulla, granulocytes, histiocytes and multinucleated giant cells and quantity of Hassall´s corpuscles were assessed (Charles, 1996; Pearse, 2006). Lymphoid depletion, i.e. reduced number/density of lymphocytes, was scored as: absent (0), mild (+), moderate (++) or severe (+++) (figure 3). The number of histiocytes was scored as: normal (0), i.e. a few individualized histiocytes

dispersed in the cortex and medulla; increased (+), i.e. many individualized and a few small clusters of histiocytes in the cortex and medulla; or markedly increased (++), i.e. many individualized and clusters of histiocytes in the cortex and medulla. Immunohistochemistry for lysozyme was used to evaluate the number of histiocytes in the thymuses (figure 4). The number of multinucleated giant cells (MGC) was scored as: none (0), few (+), some (++) or many (+++) (figure 5). The examination also included a description of other lesions, when present.

In situ hybridization

For detection of PCV2 nucleic acid, ISH with two complementary digoxigenin (DIG)-labelled oliogonucleotide probes, named complementary probe (CP) and RF probe (RFP), was performed (Rosell et al., 1999; Pérez-Martín et al., 2007). The probes were constructed from the open reading frame (ORF) 1 sequence of PCV2 encoding for the replicase proteins. The CP was complementary to ORF1 of the viral genome and hybridized with viral ssDNA, RF and mRNA, and thus detected PCV2 irrespective of replicative status. The RFP was identical to ORF1 of the viral genome and could therefore solely hybridize with the RF form, which only is present during viral replication. All thymus and bone marrow samples were investigated with the CP and the samples that were positive by this method were further examined with the RFP. The positive reactions by CP and RFP ISH were scored as low, moderate or massive and the positive cells were morphologically characterised. The ISH process for both probes was identical and was carried out according to that reported by Rosell et al. (1999). Briefly, sections were deparaffinised, rehydrated and treated with 0.3% pepsin for 10 min at 37°C, followed by heating to 105°C for 8 min. At this temperature, the sections were incubated with 100% formamide for 5 min and then prehybridized for 20 min by incubation with 0.1 nmol/ml or 0.3 nmol/ml of the CP or RFP, respectively. The hybridization process was subsequently carried out at 37°C for 60 min, where after sections were washed in high stringency bathes with saline sodium citrate. Detection was done by incubation with a 1:500 dilution of anti-DIG antibody (Roche Diagnostics, Spain) at 37°C for 60 min, followed by colour development by NBT/BCIP stock solution (Roche Diagnostics, Spain) at 37°C for 20 min. Sections were counterstained by fast green (Sigma-Aldrich, Spain) and dehydrated and coverslides were mounted before examination. A section of a lymph node or trachea from a PMWS-affected pig was the positive control for validation of the ISH process with the CP or RFP, respectively.

Statistical analyses

The Chi-square test, Fisher's exact test and/or Student's two sample t-test of SAS version 9.1 (SAS Institute, NC, USA) were used for analysing the results. Statistical significant level was set at $P<0.05$. The small group sizes of the experimentally infected pigs precluded valid statistical analysis.

Results

Histopathology associated with the primary lymphoid organs

Bone marrow

The main histopathological findings are listed in table 2. No association was found when comparing the general cellularity of PMWS cases and controls by the Chi-square test. Using the Student's two sample t-test and Fisher's exact test no significant difference or association was observed between PMWS cases and controls according to M:E ratio. When the quantity of megakaryocytes and other cells in the bone marrow was evaluated, no deviations were detected. Furthermore, no morphological abnormalities were detected in these cells. The distribution of FAEM was focal, multifocal or affected most of the bone marrow section. Adipocytes in the affected areas showed varying degrees of atrophy. Alcian blue stained the FAEM, identifying the mucopolysaccharide nature of the substance and the staining intensity had a positive association with the amount of FAEM (figure 2 b, d). The FAEM was negative by staining for collagen and reticulin. The Fisher's exact test showed that there was a strong association between FAEM and having PMWS ($P<0.0001$). Localized aggregates of Giemsa- and CD3-positive lymphocytes and lysozyme-positive histiocytes, sometimes surrounding the blood vessel, were detected in the bone marrows of 11/33 (~33%) of the PMWS-affected animals and 4/29 (~14%) of the controls from the field study (figure 6a). However, using the Chi-square test, this difference was not significant ($P=0.07$). The lymphoid aggregates were not seen in the bone marrows of the experimentally infected animals. No inclusion bodies were found in the bone marrow sections of animals from the experimental or field studies.

Thymus

The main histopathological findings are summarized in table 3. The detected lymphoid depletion primarily affected the cortical lymphocytes (figure 3). In the severely depleted thymuses, only a few Hassall´s corpuscles were detected. The MGC were mainly located in the medullary area in the

thymuses without lymphoid depletion, whereas the MGC were present in both cortex and medulla of the depleted thymuses. The Fisher's exact test showed that there was a strong association between PMWS and degree of lymphoid depletion ($P<0.0001$), number of histiocytes ($P<0.0001$) and MGC ($P=0.0002$). Inclusion bodies were not found in any of the thymuses. In all thymus sections, varying numbers of cells containing multiple small uniform, intensely eosinophilic cytoplasmatic granules were seen (figure 7a). The majority of these cells had abundant cytoplasm and contained one large, round to oval and often eccentrically located nucleus, but all intergrades of nucleus size from large to small could be seen. Mitotic figures were sometimes observed. Individualized cells were located at the cortico-medullary junction and diffusely spread in the medullary and cortical tissues, especially in the connective tissue of trabeculae and vessels. Mature eosinophilic granulocytes could also be seen adjacent to the mononuclear eosinophilic granular cells and both cell types stained positive by Luna staining (figure 7b). The proportion of eosinophilic mononuclear cells to eosinophilic granulocytes differed from pig to pig. Sometimes neutrophils and eosinophils were located in close proximity to the Hassall's corpuscles in the thymuses.

In situ hybridization

The results of CP ISH are displayed in table 4. All sections of bone marrow and thymus from the controls and PCV2-inoculated pigs of the experimental infection study were negative for PCV2 by CP ISH and this was also the case for the healthy animals from the field study. In the PMWS-affected animals, 19/20 (~95%) of the available thymuses had positive reaction of varying intensity with the CP ISH, whereas the CP ISH only gave low grade reaction in the positive bone marrows (16/33 ~48%) (figure 8a, b). Detection of PCV2 in thymus and bone marrow was associated with systemic moderate or massive PCV2 loads, detected by CP ISH in the secondary lymphoid organs (table 5). The results of RFP ISH are listed in table 6. In both thymus and bone marrow, only a few cells were positive when tested by the RFP ISH. In the thymuses, RFP ISH-positive reaction was seen exclusively in the animals that had massive PCV2 load by CP ISH (figure 8c, d). In the animals where both bone marrow and thymus were available ($n = 20$), the CP gave positive reaction in both organs of 9 (~45%) animals. Four of these 9 animals (~44%) had positive reaction in both bone marrow and thymus with the RFP, 3 (~33%) animals were only positive in thymus and the remaining 2 (~22%) animals were negative in both organs. The CP ISH labelled the cytoplasm and sometimes the nucleus of the positive cells in both thymus and bone marrow, whereas the RFP ISH only stained the nucleus of the positive cells. The CP- and RFP-positive cells in the bone marrows

were characterized as histiocyte-like cells and were primarily located in the lymphoid aggregates and perivascular tissues (figure 6b). In thymus, the CP mainly labelled the cytoplasm of histiocyte-like cells in the cortex and medulla, but cortical lymphocyte-like cells with nuclear staining could also be detected in some thymuses (figure 8b). The RFP-positive cells were seen primarily in the cortex and most of them were histiocyte-like, but positive cortical lymphocyte-like cells were also observed in some of the thymuses (figure 8d).

Discussion

Despite the huge amount of data concerning PMWS in general and the pathogenesis of PCV2 in particular, we found that there was a lack of information concerning the primary lymphoid organs. Over time, there have been speculations about whether the primary lymphoid organs have an important position in the pathogenesis of PCV2 and if the cell types permissive of replication should be found here (Darwich et al., 2004).

Like others (Yu et al., 2007; Pérez-Martín et al., 2007), we have considered if the PCV2 replication takes place early in the infection, even before clinical signs develop and therefore materials from experimentally PCV2-inoculated animals were examined. In the original experimental infection study, Fernandes et al. (2007) found PCV2-positive lymph nodes by CP ISH, but no PCV2 DNA could be detected in the thymus or bone marrow from these animals in the present study. This is in contrast with previous experimental infection studies, where PCV2 was detected by IHC in thymus (bone marrow not examined) from specific pathogen free piglets (Magar et al., 2000) and conventional pigs (Yu et al., 2007). Since the experimental infection study by Fernandes et al. (2007) did not reproduce clinical PMWS, it was not regarded as a successful model of PMWS by Tomás et al. (2008). According to this paper, the best way of achieving success in an experimental PMWS study was to use colostrum-deprived piglets younger than 3 weeks of age and to inoculate with high doses ($>10^5$ $TCID_{50}$/pig) of PCV2 from genotype b (Segalés et al., 2008) together with another porcine pathogen. The experimentally infected piglets in the present study were only inoculated with a PCV2 strain of genotype a (Fernandes et al., 2007; Segalés et al., 2008), which might partially explain why PCV2 was not detected in the thymus and bone marrow. Taking this into consideration, it would be worth examining thymus and bone marrow in another experimental setting before the role of primary lymphoid organs in the initial pathogenesis of the PCV2 infection can be concluded.

In the PMWS-affected pigs, the CP ISH gave positive reaction in 95% of the thymuses and 48% of the bone marrows. In the animals where both bone marrow and thymus were available, 45% had PCV2 in both organs. The PCV2 load in these animals was moderate or massive in most (67%) thymuses and low in all bone marrows. In general, the finding of PCV2 (CP) in thymus and bone marrow was associated with high amounts of PCV2 by CP ISH in the secondary lymphoid organs. This could be interpreted as a spill over to thymus and bone marrow, when the general virus load in the secondary lymphoid organs was high. Since almost all thymuses from the PMWS cases and only half of the bone marrows were positive for PCV2 by CP ISH and the virus load in the thymus in general was higher compared to that of the bone marrow, this could indicate that thymus is more susceptible or bone marrow more resistant to PCV2 infection. PCV2 replication was detected in 37% of the thymuses and 31% of the bone marrows from those tissues that gave positive results by CP ISH, indicating that the ratio of replication was similar in these two organs. In the bone marrow, the cell type positive for PCV2 nucleic acids and signs of viral replication morphologically resembled histiocytes. No PCV2 or PCV2 replication was found in bone marrow specific cells, indicating that the bone marrow is not a specific target organ in regard of PCV2 infection. In the thymus, PCV2 nucleic acids were primarily found in the cytoplasm of histiocyte-like cells throughout the cortex and medulla; however, PCV2-positive lymphocyte-like cells with nuclear staining were also detected in the cortex. Furthermore, viral replication was found to be associated with massive amounts of PCV2 in the thymus. Most of the RFP-positive cells in the thymus were histiocytes, mainly located in cortex, but cortical lymphocyte-like cells also supported PCV2 replication. The results support the general accepted idea that histiocytes are the main cell type responsible for passive accumulation and systemic spread of PCV2 (Darwich et al., 2004; Opriessnig et al., 2007). Furthermore it was found that histiocytes of the thymus and bone marrow, and thymic lymphocytes support viral replication, though it was not possible to tell whether the cortical lymphocytes were thymocytes or lymphocytes migrating through thymus. This is in agreement with previous reports by Pérez-Martín et al. (2007), Yu et al. (2007) and Hamberg et al. (2007), who could not link PCV2 replication to a specific cell type, but found that replication was supported by different cell types, including histiocytes and lymphocytes. In the present study, viral replication was only detected in a few scattered cells in both thymus and bone marrow. An explanation for this could be that the tissue/organ supporting the PCV2 replication has not yet been identified. It has become known that in several mammals, including swine, the ileal Peyer's patch

(IPP) has the function of a primary lymphoid organ and is a mammalian homologue to the avian bursa of Fabricius (Butler et al., 2006; Yasuda et al., 2006; Butler and Sinkora, 2007). Furthermore, the bursa of Fabricius, a major supplier of B-lymphocytes (Yasuda et al., 2006) is affected by the avian circoviruses (Coletti et al., 2000; Todd, 2000; Smyth et al., 2005). In PMWS, the associated lymphopenia and lymphoid depletion affect B-lymphocytes before T-lymphocytes (Nielsen et al., 2003; Sarli et al., 2001) and the B-lymphocytes also seem to be more susceptible to PCV2 infection (Yu et al., 2007; Lin et al., 2008). Therefore any interference with the function of the IPP might influence the total number of B-lymphocytes in vivo and cause at least part of the reduction in B-lymphocytes seen in PMWS.

The FAEM in the bone marrow stained positive by Alcian blue and this indicates the presence of acid mucopolysaccharides, which is not seen in normal bone marrow (Bohm, 2000). Serous fat atrophy of bone marrow is characterised macroscopically by the presence of gelatinous substance and histologically by the finding of atrophic adipocytes, marrow hypoplasia and deposition of acid mucopolysaccharides, which are visible on HE stainings as an eosinophilic ground substance (Cornbleet et al., 1977; Bohm, 2000; Bollen and Skydsgaard, 2006). In the present study, it is believed that the FAEM in the bone marrow resembled serous fat atrophy, based on the combination of the appropriate histopathology and presence of acidic mucopolysaccarides, though no macroscopical signs of serous fat atrophy were seen during necropsy. The lack of macroscopical findings could be explained by the fact that the pigs were euthanized before they became cachectic. Serous fat atrophy is seen in many different conditions and can therefore not be linked to a specific disease, but when present, it indicates that the disease is severe (Bohm, 2000). The FAEM score was higher ($P<0.0001$) in the PMWS-affected animals, compared to the control animals. This difference between PMWS-affected and control pigs is consistent with the fact that reduced weight gain and wasting are some of the general clinical signs of PMWS (Chae, 2004; Opriessnig et al., 2007). To our knowledge serous fat atrophy in bone marrow has not been described before in relation to PMWS.

In the bone marrows, the CP and RFP positive cells were often detected in lymphoid aggregates, which are referred to as lymphoid nodules (Thiele et al., 1999) or reactive lymphoid lesions (Diebold et al., 2000). Reactive lymphoid lesions are composed of small clusters of B- and T-lymphocytes and few histiocytes, and can be seen in the bone marrow in relation to chronic

inflammation and viral infections (Thiele et al., 1999; Diebold et al., 2000). Although not significant (P=0.07), the finding of reactive lymphoid lesions in the bone marrow of the PMWS-affected animals correlates well with the chronic nature of PMWS. To our knowledge, reactive lymphoid lesions in the bone marrow of PMWS-affected pigs have not been described in previous studies.

The mean M:E ratios of the bone marrow of PMWS cases and control animals were in general lower than the previously reported porcine M:E ratio of 1.77 ± 0.52 :1 (Thorn, 2000). This can rely on the method used for examination of the M:E ratio, since evaluation based on biopsies only gives coarse estimates of the M:E ratio compared to fluorescence activated cell sorter analysis or differential counting of bone marrow aspirates (Cotelingam, 2003). There was not found any association between PMWS and M:E ratio or changes in the general cellularity; thus, in the present study there was no evidence that PCV2 specifically affects the myeloid or erythroid cell lines in the bone marrow.

In the thymus, the significant findings of lymphoid depletion (P<0.0001) and infiltration of histiocytes (P<0.0001) and MGC (P=0.0002) in cases of PMWS, compared to the control animals, correlate with previous reports (Chae, 2004; Segalés et al., 2005). Moreover, all thymuses contained varying numbers of cells with multiple uniform eosinophilic granules, in which some were large mononuclear cells with abundant cytoplasm. Thymus eosinophila has been described in humans (Lee et al., 1995; Jacobsen et al., 2007), mice (Muller, 1977; Jacobsen et al., 2007) and pigs (Badertscher, 1920; Rosario et al., 1995; Charles, 1996). The presence of eosinophilic precursors in the human thymus is a normal finding (Lee et al., 1995; Flores et al., 1999), but besides a study by Badertscher (1920) it has not been possible to find any descriptions of eosinophilic precursors in the thymus of pigs. Based on the morphology, positive Luna staining and the descriptions from the human thymus, it is believed that the eosinophilic cells seen in the porcine thymus were eosinophilic precursors. Since the exact function of these thymic mature and immature eosinophilic granulocytes in humans is still obscure (Flores et al., 1999; Jacobsen et al., 2007), it might be beneficial to use the pig as a model to elucidate the role of the thymic eosinophilic granulocytes. The eosinophilic precursors seemed not to be associated with PMWS, since the quantity and distribution were the same in PMWS cases and controls.

In conclusion, the primary lymphoid organs are definitively affected by PCV2 infection, but in the present study there was no evidence for thymus or bone marrow being apparently important in regard to PCV2 replication in cases of PMWS.

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References

Badertscher, J.A., 1920. Eosinophilic leucocytes in the thymus of postnatal pigs. Anat. Rec. 18, 23-34.

Biagini, P., Todd, D., Bendinelli, M., Hino, S., Mankertz, A., Mishiro, S., Niel, C., Okamoto, H., Raidal, S., Ritchie, B.W., Teo, G.C., 2005. Anellovirus. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 335-341.

Bohm, J., 2000. Gelatinous transformation of the bone marrow: the spectrum of underlying diseases. Am. J. Surg. Pathol. 24, 56-65.

Bollen, P., Skydsgaard, M., 2006. Restricted feeding may induce serous fat atrophy in male Gottingen minipigs. Exp. Toxicol. Pathol. 57, 347-349.

Bradbury, P., Rae, K., 1996. Connective tissues and stains. In: Bancroft, J.D., Stevens, A. (Eds.), Theory and practice of Histological Techniques. Churchill Livingstone, New York, pp. 113-138.

Butler, J.E., Sinkora, M., 2007. The isolator piglet: a model for studying the development of adaptive immunity. Immunol. Res. 39, 33-51.

Butler, J.E., Sun, J., Wertz, N., Sinkora, M., 2006. Antibody repertoire development in swine. Dev. Comp. Immunol. 30, 199-221.

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. Vet. J. 168, 41-49.

Charles, J.A., 1996. Lymph Nodes and Thymus. In: Sims, L.D., Glastonbury, J.R.W. (Eds.), Pathology of the Pig. A Diagnostic Guide. The Pig Research and Development Corporation and Agriculture Victoria, Victoria, pp. 185-210.

Chianini, F., Majo, N., Segalés, J., Dominguez, J., Domingo, M., 2001. Immunohistological study of the immune system cells in paraffin-embedded tissues of conventional pigs. Vet. Immunol. Immunopathol. 82, 245-255.

Coletti, M., Franciosini, M.P., Asdrubali, G., Passamonti, F., 2000. Atrophy of the primary lymphoid organs of meat pigeons in Italy associated with circoviruslike particles in the bursa of Fabricius. Avian Dis. 44, 454-459.

Cook, H.C., 1996. Carbohydrates. In: Bancroft, J.D., Stevens, A. (Eds.), Theory and practice of Histological Techniques. Churchill Livingstone, New York, pp. 173-212.

Cornbleet, P.J., Moir, R.C., Wolf, P.L., 1977. A histochemical study of bone marrow hypoplasia in anorexia nervosa. Virchows Arch. A Pathol. Anat. Histol. 374, 239-247.

Cotelingam, J.D., 2003. Bone marrow biopsy: interpretive guidelines for the surgical pathologist. Adv. Anat. Pathol. 10, 8-26.

Cramer, A.D., Rogers, E.R., Parker, J.W., Lukes, R.J., 1973. The Giemsa stain for tissue sections: an improved method. Am. J. Clin. Pathol. 60, 148-156.

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.

Diebold, J., Molina, T., Camilleri-Broet, S., Le, T.A., Audouin, J., 2000. Bone marrow manifestations of infections and systemic diseases observed in bone marrow trephine biopsy review. Histopathology 37, 199-211.

Elmore, S.A., 2006. Enhanced histopathology of the thymus. Toxicol. Pathol. 34, 656-665.

Fernandes, L.T., Mateu, E., Sibila, M., Fort, M., Andaluz, A., McNeilly, F., Allan, G., Sanchez, A., Segalés, J., Stevenson, L., 2007. Lack of in vitro and in vivo effects of lipopolysaccharide on porcine circovirus type 2 infection. Viral Immunol. 20, 541-552.

Flores, K.G., Li, J., Sempowski, G.D., Haynes, B.F., Hale, L.P., 1999. Analysis of the human thymic perivascular space during aging. J. Clin. Invest. 104, 1031-1039.

Fort, M., Fernandes, L.T., Nofrarias, M., Diaz, I., Sibila, M., Pujols, J., Mateu, E., Segalés, J., 2009. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. Vet. Immunol. Immunopathol., doi: 10.1016/j.vetimm.2008.12.024.

Ghebremariam, M.K., Gruys, E., 2005. Postweaning multisystemic wasting syndrome (PMWS) in pigs with particular emphasis on the causative agent, the mode of transmission, the diagnostic tools and the control measures. A review. Vet. Q. 27, 105-116.

Grau-Roma, L., Hjulsager, C.K., Sibila, M., Kristensen, C.S., López-Soria, S., Enøe, C., Casal, J., Bøtner, A., Nofrarías, M., Bille-Hansen, V., Fraile, L., Baekbo, P., Segalés, J., 2008. Infection, excretion, seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from postweaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark. Vet. Microbiol., doi:10.1016/j.vetmic.2008.10.007.

Hamberg, A., Ringler, S., Krakowka, S., 2007. A novel method for the detection of porcine circovirus type 2 replicative double stranded viral DNA and nonreplicative single stranded viral DNA in tissue sections. J. Vet. Diagn. Invest. 19, 135-141.

Harvey, J.W., 2001. Atlas of veterinary hematology. Blood and bone marrow of domestic animal. W.B. Saunders Company, Philadelphia.

Hino, S., Miyata, H., 2007. Torque teno virus (TTV): current status. Rev. Med. Virol. 17, 45-57.

Jacobsen, E.A., Taranova, A.G., Lee, N.A., Lee, J.J., 2007. Eosinophils: singularly destructive effector cells or purveyors of immunoregulation? J. Allergy Clin. Immunol. 119, 1313-1320.

Kakkola, L., Tommiska, J., Boele, L.C., Miettinen, S., Blom, T., Kekarainen, T., Qiu, J., Pintel, D., Hoeben, R.C., Hedman, K., Soderlund-Venermo, M., 2007. Construction and biological activity of a full-length molecular clone of human Torque teno virus (TTV) genotype 6. FEBS J. 274, 4719-4730.

Kohler, H., 1956. Knochenmark und Blutbild des Ferkels. 1. Mitteilung: Das gesunde Ferkel. Zentralbl. Veterinärmed. Reihe A 3, 359-395.

Kvist, P.H., Iburg, T., Bielecki, M., Gerstenberg, M., Buch-Rasmussen, T., Hasselager, E., Jensen, H.E., 2006. Biocompatibility of electrochemical glucose sensors implanted in the subcutis of pigs. Diabetes Technol. Ther. 8, 463-475.

Lee, I., Yu, E., Good, R.A., Ikehara, S., 1995. Presence of eosinophilic precursors in the human thymus: evidence for intra-thymic differentiation of cells in eosinophilic lineage. Pathol. Int. 45, 655-662.

Lin, C.M., Jeng, C.R., Chang, H.W., Guo, I.C., Huang, Y.L., Tsai, Y.C., Chia, M.Y., Pang, V.F., 2008. Characterization of porcine circovirus type 2 (PCV2) infection in swine lymphocytes using mitogen-stimulated peripheral blood lymphocytes from healthy PCV2-carrier pigs. Vet. Immunol. Immunopathol. 124, 355-366.

Luna, L.G., 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. McGraw-Hill Book Company, New York.

Magar, R., Larochelle, R., Thibault, S., Lamontagne, L., 2000. Experimental transmission of porcine circovirus type 2 (PCV2) in weaned pigs: a sequential study. J. Comp. Pathol. 123, 258-269.

Marlier, D., Vindevogel, H., 2006. Viral infections in pigeons. Vet. J. 172, 40-51.

Muller, E., 1977. Localization of eosinophils in the thymus by the peroxidase reaction. Histochemistry 52, 273-279.

Nielsen, J., Vincent, I.E., Botner, A., Ladekaer-Mikkelsen, A.S., Allan, G., Summerfield, A., McCullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. 92, 97-111.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Invest. 19, 591-615.

Pearse, G., 2006. Normal Structure, Function and Histology of the Thymus. Toxicol. Pathol. 34, 504-514.

Pérez-Martín, E., Rovira, A., Calsamiglia, M., Mankertz, A., Rodriguez, F., Segalés, J., 2007. A new method to identify cell types that support porcine circovirus type 2 replication in formalin-fixed, paraffin-embedded swine tissues. J. Virol. Methods 146, 86-95.

Rosario, P., Loris, Z., Giuseppe, S., Claudia, E., Stefano, M.P., 1995. Quantitative determination of thymic eosinophilia in swine. Ital. J. Anat. Embryol. 100, 171-178.

Rosell, C., Segalés, J., Plana-Duran, J., Balasch, M., Rodriguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J. Comp. Pathol. 120, 59-78.

Sarli, G., Mandrioli, L., Laurenti, M., Sidoli, L., Cerati, C., Rolla, G., Marcato, P.S., 2001. Immunohistochemical characterisation of the lymph node reaction in pig post-weaning multisystemic wasting syndrome (PMWS). Vet Immunol. Immunopathol. 83, 53-67.

Segalés, J., 2002. Update on postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome diagnostics. J. Swine Health Prod. 10, 277-281.

Segalés, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. Anim. Health Res. Rev. 6, 119-142.

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, 867-868.

Smyth, J., Soike, D., Moffett, D., Weston, J.H., Todd, D., 2005. Circovirus-infected geese studied by in situ hybridization. Avian Pathol. 34, 227-232.

Sorden, S.D., 2000. Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). Swine Health Prod. 8, 133-136.

Stevens, A., Wilson, I.G., 1996. The haematoxylins and eosin. In: Bancroft, J.D., Stevens, A. (Eds.), Theory and practice of Histological Techniques. Churchill Livingstone, New York, pp. 99-112.

STP Immunotoxicology Working Group, Haley, P., Perry, R., Ennulat, D., Frame, S., Johnson, C., Lapointe, J.M., Nyska, A., Snyder, P.W., Walker, D., Walter, G., 2005. STP Position Paper: Best Practice Guideline for the Routine Pathology Evaluation of the Immune System. Toxicol. Pathol. 33, 404-407.

Thiele, J., Zirbes, T.K., Kvasnicka, H.M., Fischer, R., 1999. Focal lymphoid aggregates (nodules) in bone marrow biopsies: differentiation between benign hyperplasia and malignant lymphoma--a practical guideline. J. Clin. Pathol. 52, 294-300.

Thorn, C.E., 2000. Normal Hematology of the Pig. In: Feldman, B.F., Zinkl, J.G., Jain, N.C. (Eds.), Schalm´s Veterinary Hematology. Lippincott Williams & Wilkins, Baltimore, pp. 1089-1095.

Todd, D., 2000. Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathol. 29, 373-394.

Todd, D., Bendinelli, M., Biagini, P., Hino, S., Mankertz, A., Mishiro, S., Niel, C., Okamoto, H., Raidal, S., Ritchie, B.W., Teo, G.C., 2005. Circoviridae. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 327-334.

Tomas, A., Fernandes, L.T., Valero, O., Segalés, J., 2008. A meta-analysis on experimental infections with porcine circovirus type 2 (PCV2). Vet. Microbiol. In press.

Yasuda, M., Jenne, C.N., Kennedy, L.J., Reynolds, J.D., 2006. The sheep and cattle Peyer's patch as a site of B-cell development. Vet. Res. 37, 401-415.

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.

Tables

Table 1
Histochemical and immunohistochemical staining procedures applied on thymus and bone marrow

Staining method	Items examined	Tissue and no. examined	Reference
Histochemical staining			
Hematoxylin and eosin	General morphology	BM[1] (all), thymus (all)	(Stevens and Wilson, 1996)
Masson trichrome	Collagene	BM (14)	(Luna, 1968) modified
Gordon & Sweet reticulin	Reticulin	BM (19)	(Bradbury and Rae, 1996)
Alcian blue (pH 3)	Acid mucopolysaccarides	BM (25)	(Cook, 1996) modified
Periodic acid-shiff	[2]HP & BC, [3]M:E ratio	BM (all)	(Cook, 1996) modified
Giemsa	HP & BC, M:E ratio	BM (all)	(Cramer et al., 1973) modified
Luna	Eosinophils	Thymus (22)	(Luna, 1968)
Immunohistochemical staining			
Lysozyme[4]	Histiocytes	BM (3), thymus (22)	(Chianini et al., 2001) modified
CD3[5]	T-lymphocytes	BM (3)	(Kvist et al., 2006)

[1]BM = bone marrow. [2]HP & BC = hematopoietic precursors and blood cells. [3] M:E ratio = myeloid to erythroid ratio.
[4]Antibody: a 1:350 dilution of polyclonal rabbit anti-humane lysozyme antibody (A0099) (DakoCytomation, Glostrup, Denmark). Antigen retrieval: 2 h in 0.1% trypsin at 37°C. Detection: EnVision/HRP (K4003) (DakoCytomation, Glostrup, Denmark). Chromogene: DAB (KemEnTec, Taastrup, Denmark).
[5]Antibody: a 1:1000 dilution of monoclonal mouse anti-porcine CD3ε antibody (4510-01) (SouthernBiotech, Alabama, USA). Antigen retrieval: 2x5 min. in microwave oven (700 watts) in Tris-EGTA buffer (pH 9), cool 15 min. Detection: EnVision/HRP (K4001) (DakoCytomation, Denmark). Chromogene: DAB (KemEnTec, Taastrup, Denmark).

Table 2
The major histopathological findings and myeloid to erythroid ratios in bone marrow

	Experimental infection study						*Field study*	
	Control			PCV2[1]-inoculated			Control	PMWS[2]-affected
	DPI[3] 5 (n=2)	8 (n=2)	29 (n=8)	DPI 5 (n=6)	8 (n=7)	29 (n=11)	(n=29)	(n=33)
Hypocellularity[4]								
0	2 (100%)	1 (50%)	8 (100%)	3 (50%)	6 (86%)	10 (91%)	3 (10%)	8 (24%)
+	0	1 (50%)	0	3 (50%)	1 (14%)	1 (9%)	8 (28%)	7 (21%)
++	0	0	0	0	0	0	7 (24%)	13 (39%)
+++	0	0	0	0	0	0	11 (38%)	5 (15%)
M:E ratio[5]								
0.8-1.1	0	2 (100%)	0	0	2 (29%)	0	5 (20%)	3 (9%)
1.2-1.5	1 (50%)	0	4 (50%)	3 (50%)	2 (29%)	5 (45%)	3 (12%)	6 (19%)
1.6-1.9	1 (50%)	0	4 (50%)	2 (33%)	3 (43%)	4 (36%)	11 (44%)	12 (38%)
2.0-2.3	0	0	0	1 (17%)	0	2 (18%)	6 (24%)	6 (19%)
2.4-2.7	0	0	0	0	0	0	0	3 (9%)
2.8-3.1	0	0	0	0	0	0	0	2 (6%)
Mean	1.55	1.05	1.55	1.63	1.56	1.63	1.63	1.82
SD	0.35	0.07	0.21	0.25	0.96	0.29	0.36	0.55
FAEM[6]								
0	2 (100%)	1 (50%)	8 (100%)	4 (67%)	7 (100%)	11(100%)	25 (86%)	7 (21%)
+	0	1 (50%)	0	2 (33%)	0	0	4 (14%)	8 (24%)
++	0	0	0	0	0	0	0	13 (39%)
+++	0	0	0	0	0	0	0	5 (15%)
Lymphoid aggregates								
	0	0	0	0	0	0	4 (14%)	11 (33%)

[1]PCV2 = porcine circovirus type 2. [2]PMWS = postweaning multisystemic wasting syndrome. [3]DPI = days post inoculation.
[4]Hypocellularity. General marrow cellularity was graded as follows: normocellular (0), slightly hypocellular (+), hypocellular (++) or very hypocellular/acellular (+++)
[5]Myeloid to erythroid ratio (M:E ratio) was divided into categories and the data are shown as mean for each group of animals and corresponding standard deviation (SD). In the spontaneous infection study 4 bone marrows from the control animals and one bone marrow from a PMWS-affected animal were acellular, preventing evaluation.
[6]The amount of fat associated eosinophilic material (FAEM) was graded as follows: absent (0), low (+), moderate (++) or high (+++).

Table 3
The major histopathological findings in thymus

	Experimental infection study						*Field study*	
	Control			PCV2[1]-inoculated			Control	PMWS[2]-affected
	DPI[3] 5 (n=2)	8 (n=2)	29 (n=7)	DPI 5 (n=6)	8 (n=7)	29 (n=11)	(n=28)	(n=20)
Lymphoid depletion[4]								
0	1 (50%)	1 (50%)	6 (86%)	5 (83%)	6 (86%)	8 (73%)	26 (93%)	7 (35%)
+	0	1 (50%)	1 (14%)	1 (17%)	0	3 (27%)	2 (7%)	1 (5%)
++	1 (50%)	0	0	0	1 (14%)	0	0	8 (40%)
+++	0	0	0	0	0	0	0	4 (20%)
Histiocytes[5]								
0	2 (100%)	2 (100%)	7 (100%)	6 (100%)	7 (100%)	9 (82%)	20 (71%)	3 (15%)
+	0	0	0	0	0	2 (18%)	8 (29%)	10 (50%)
++	0	0	0	0	0	0	0	7 (35%)
Multinucleated giant cells[6]								
0	0	1 (50%)	0	2 (33%)	5 (71%)	2 (18%)	3 (11%)	1 (5%)
+	2 (100%)	1 (50%)	6 (86%)	4 (67%)	2 (29%)	5 (45%)	21 (75%)	7 (35%)
++	0	0	1(14%)	0	0	3 (27%)	4 (14%)	3 (15%)
+++	0	0	0	0	0	1 (9%)	0	9 (45%)

[1]PCV2 = porcine circovirus type 2. [2]PMWS = postweaning multisystemic wasting syndrome. [3]DPI = days post inoculation.
[4]Lymphoid depletion was graded as follows: no (0), mild (+), moderate (++) or marked (+++).
[5]Relative number of histiocytes was graded as follows: normal (0), increased (+) or significantly increased (++).
[6]Relative number of multinucleated giant cells was graded as follows: none (0), few (+), some (++) or many (+++).

Table 4
Detection of porcine circovirus type 2 (PCV2) by in situ hybridization (ISH) with the complementary probe (CP) on thymus and bone marrow samples from control and postweaning multisystemic wasting syndrome (PMWS)-affected pigs

	Thymus		Bone marrow	
	Control (n = 28)	PMWS-affected (n = 20)	Control (n = 29)	PMWS-affected (n = 33)
CP ISH[1]				
0	28(100%)	1 (5%)	29 (100%)	17 (52%)
+	0	10 (50%)	0	16 (48%)
++	0	2 (10%)	0	0
+++	0	7 (35%)	0	0

[1]The level of PCV2 nucleic acids detected in each tissue section was graded as follows: absent (0), low (+), moderate (++) or massive (+++).

Table 5
Comparisons of porcine circovirus type 2 (PCV2) load among the secondary lymphoid organs, thymus and bone marrow of control and postweaning multisystemic wasting syndrome-affected pigs from the field study by in situ hibridization with complementary probe

		PCV2 load in thymus				PCV2 load in bone marrow			
		0	+	++	+++	0	+	++	+++
PCV2 load in	0	12*				12*			
secondary	+	16*				17*			
lymphoid	++	1	7			11	2		
organs	+++		3	2	7	6	14		

The level of PCV2 nucleic acids detected in each tissue section was graded as follows: absent (0), low (+), moderate (++) or massive (+++). Numbers refer to number of animals in each category.
*Control animals.

Table 6
Detection of porcine circovirus type 2 (PCV2) replication, by in situ hybridization (ISH) with the replicative form probe (RFP) on samples of thymus and bone marrow, which were positive by the complementary probe (CP)

	Thymus	Bone marrow
	CP ISH positive (n =19)	CP ISH positive (n = 16)
RFP ISH[1]		
0	12 (63%)	11 (69%)
+	7 (37%)	5 (31%)

[1]Replication was only detected in low levels in thymus and bone marrow.

Figure legends

Fig. 1. (a) Histology of normocellular bone marrow (grade 0). (b) Histology of very hypocellular bone marrow (grade +++). Hematoxylin and eosin stain (scale bars = 100 μm).

Fig. 2. (a) Histology of bone marrow with low amount of "fat associated eosinophilic material" (FAEM) (grade +). Hematoxylin and eosin (HE) stain. (b) Section from same bone marrow stained with Alcian blue. (c) Histology of bone marrow with high amount of FAEM (grade +++). HE stain. (d) Section from same bone marrow stained with Alcian blue. (Scale bars = 50 μm).

Fig. 3. (a) Histology of normal thymus without lymphoid depletion (grade 0). Clear distinction between cortex and medulla. (b) Mild lymphoid depletion (grade +) in the thymus of a postweaning multisystemic wasting syndrome (PMWS)-affected pig. (c) Severe lymphoid depletion (grade ++) with infiltration of histiocytes and multinucleated giant cells in the thymus of a PMWS-affected pig. Border between medulla and cortex is indistinct. Hematoxylin and eosin stain (scale bars = 100 μm).

Fig. 4. (a) Histology of thymus with normal population of histiocytes, i.e. few individualized histiocytes dispersed in the cortex and medulla (grade 0). (b) Thymus from a postweaning multisystemic wasting syndrome-affected pig with significantly increased individualized and clusters of histiocytes in the cortex and medulla. Immunohistochemistry for lysozyme (scale bars = 100 μm).

Fig. 5. Histology of thymus with many multinucleated giant cells (grade +++) and histiocytes from a postweaning multisystemic wasting syndrome affected pig. Hematoxylin and eosin stain (scale bar = 50 μm).

Fig. 6. Histologic appearance of a lymphoid aggregate associated to a blood vessel in the bone marrow; (a) immunohistochemistry for CD3; (b) in situ hybridiztion for porcine circovirus type 2 with complementary probe, chromogen NBT/BCIP and fast green counterstain. (Scale bars = 50 μm).

Fig. 7. Histologic appearance of eosinophilic granulocytes and mononuclear cells with eosinophilic granules located within the connective tissue of thymus; (a) hematoxylin and eosin stain ; (b) Luna stain. (Scale bars = 30 μm).

Fig. 8. In situ hybridiztion (ISH) for porcine circovirus type 2. (a) ISH of bone marrow with complementary probe (CP), showing cytoplasmatic (arrowhead) and nuclear (arrow) staining of histiocyte-like cells (scale bar = 20 μm). (b) CP ISH of thymus showing cytoplasmatic staining of histiocyte-like cells (arrowhead) and nuclear staining of cortical lymphocyte-like cells (arrow) (scale bar = 30 μm). Inset: CP ISH of thymus showing cytoplasmatic staining of multinucleated giant cell (scale bar = 20 μm). (c) ISH of bone marrow with replicative form probe (RFP), showing nuclear staining of a histiocyte-like cell (scale bar = 20 μm). (d) RFP ISH of thymus showing nuclear staining of three histiocyte-like cells in medulla (scale bar = 20 μm). Inset: RFP ISH of thymus showing nuclear staining of a cortical lymphocyte-like cell (scale bar = 20 μm). Chromogen NBT/BCIP and fast green counterstain.

Figure 1

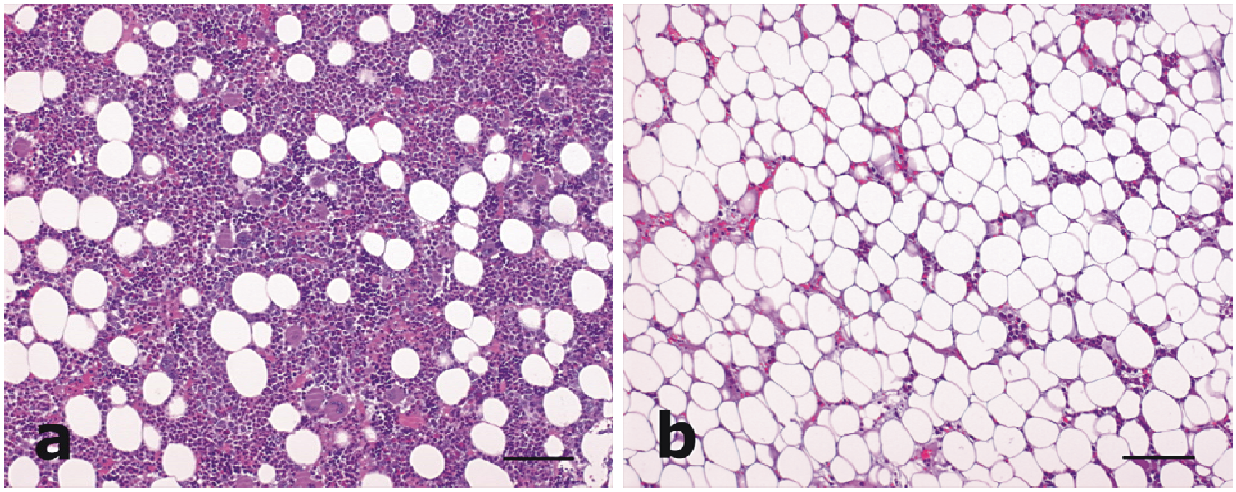


Figure 2

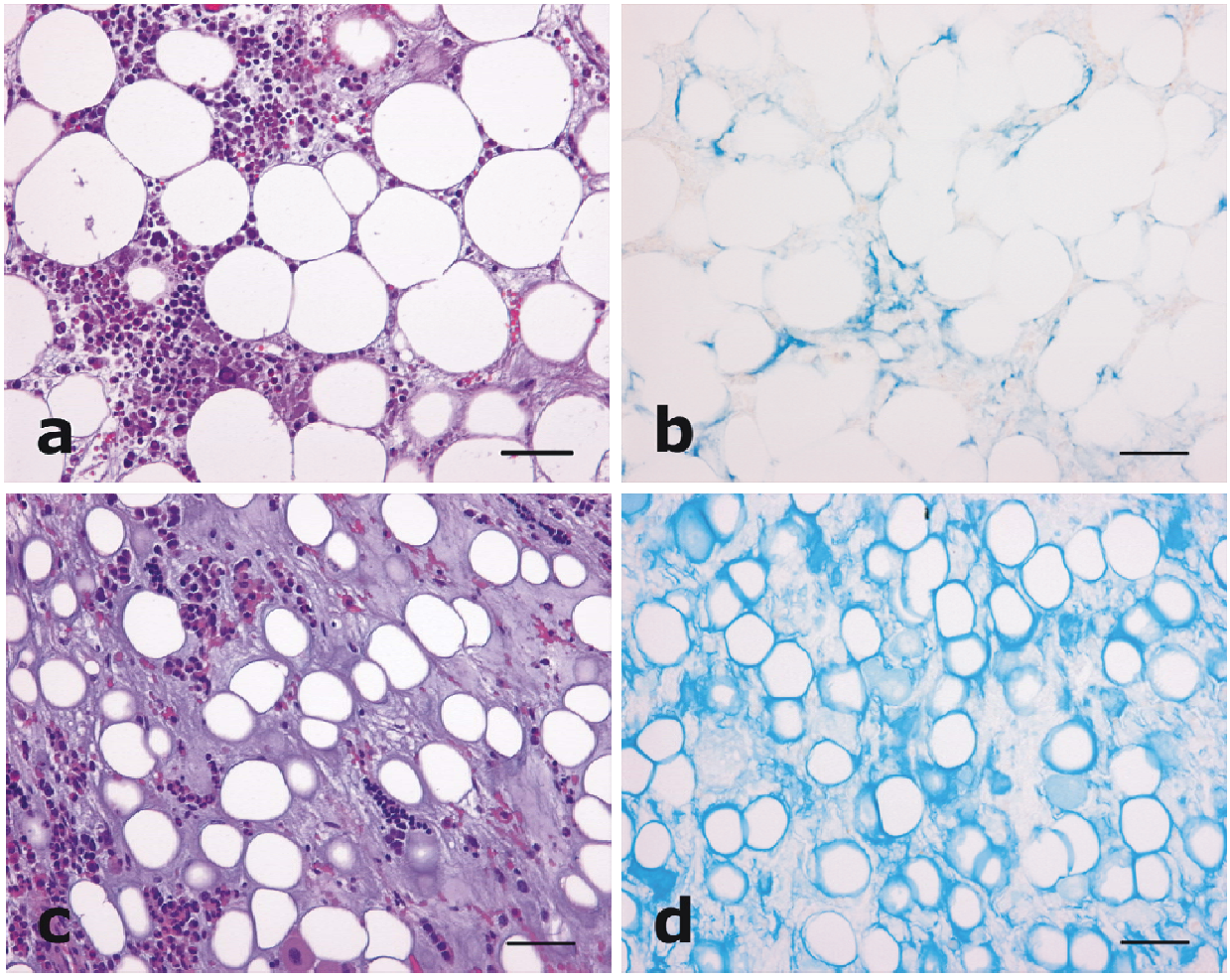


Figure 3

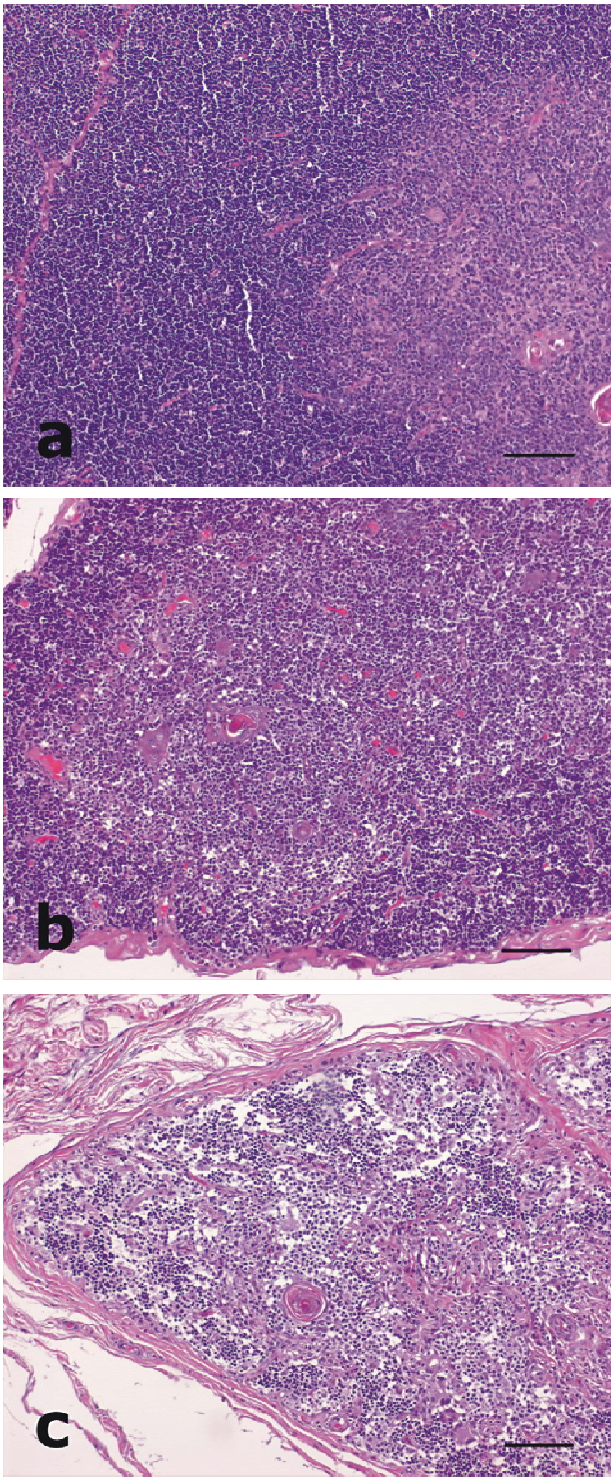


Figure 4

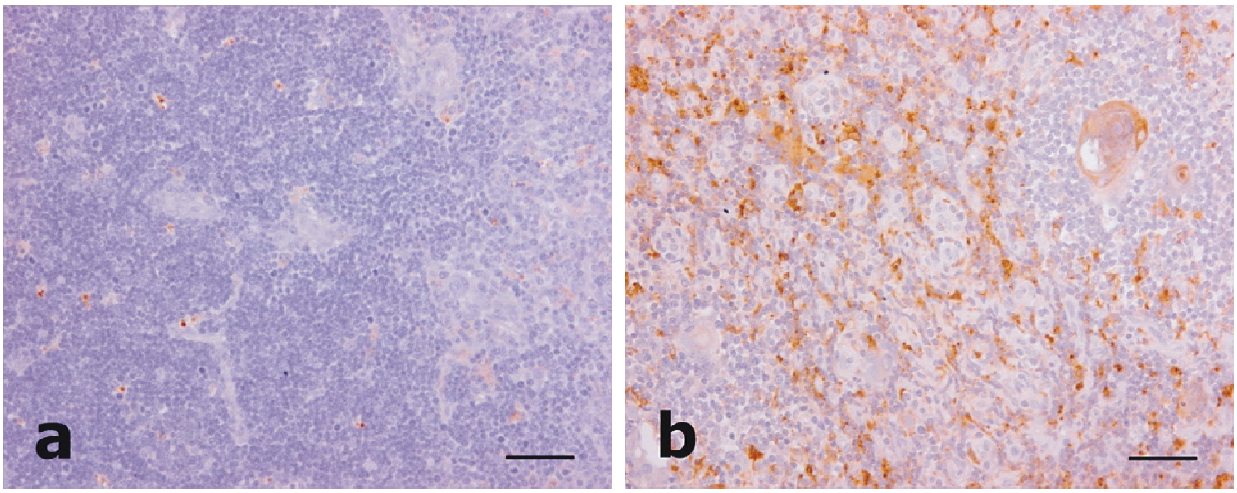


Figure 5

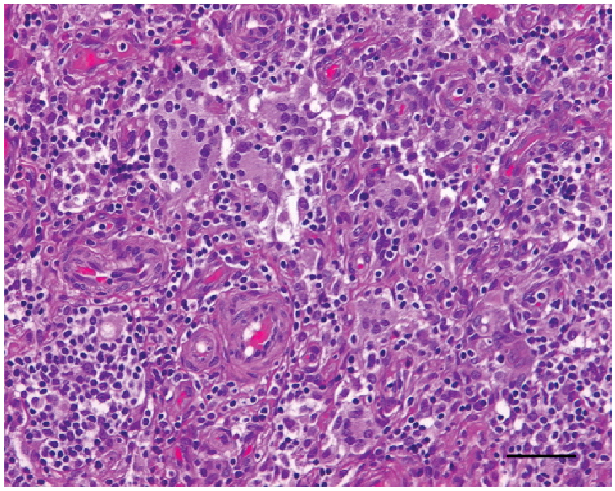


Figure 6

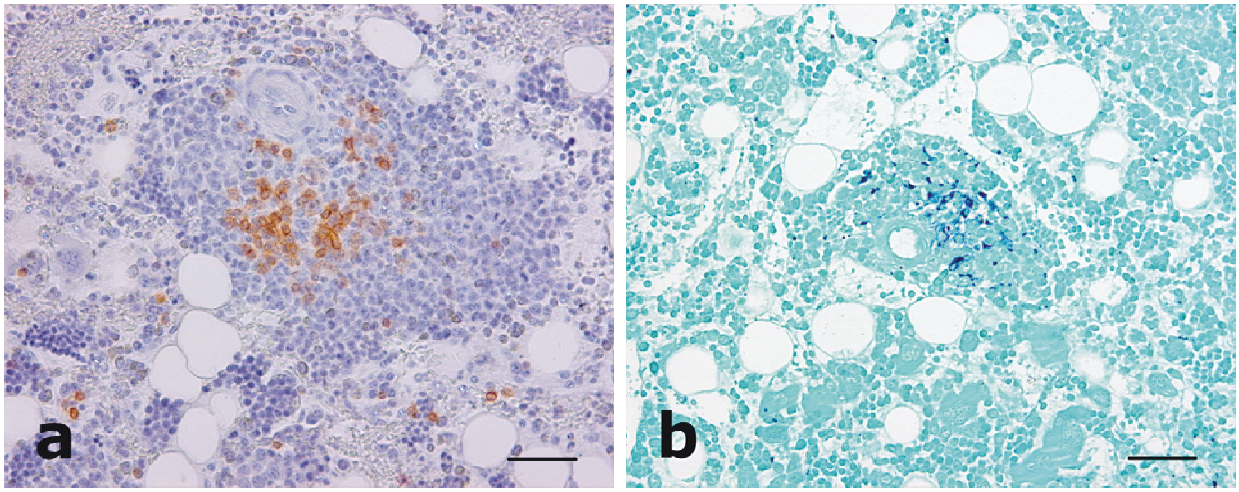


Figure 7

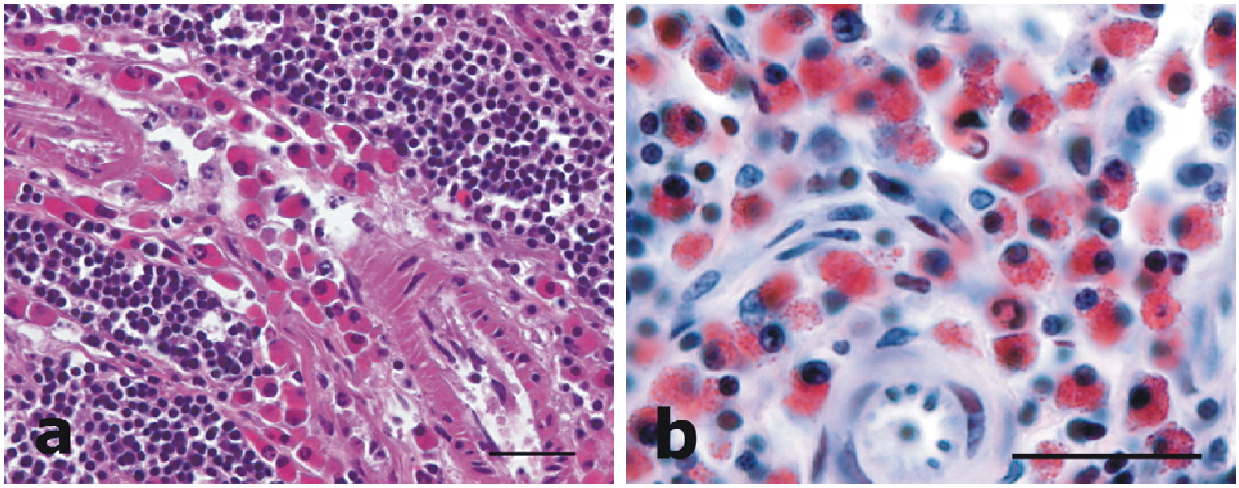
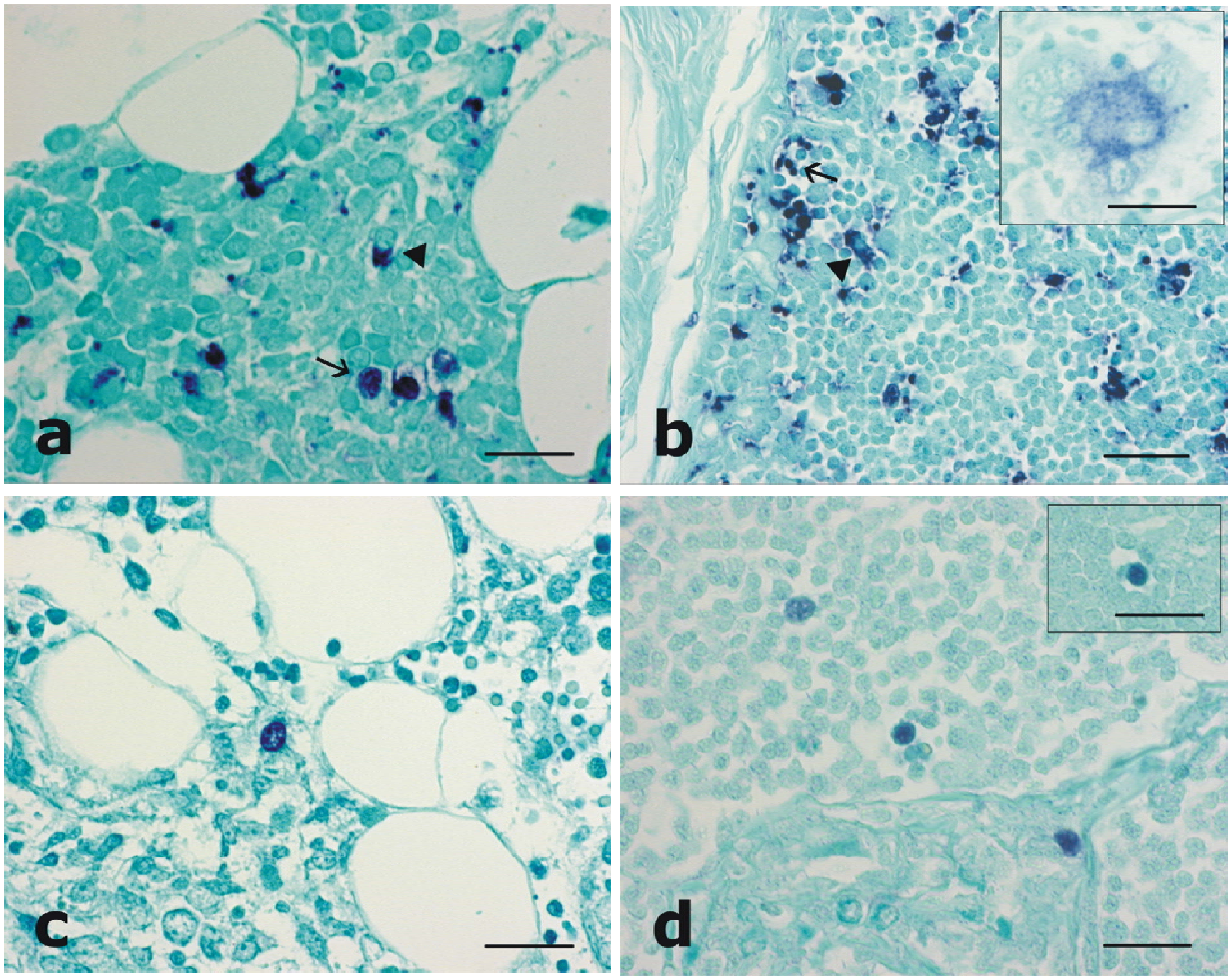


Figure 8



Manuscript II

Occurrence and tissue distribution of porcine circovirus type 2 identified by immunohistochemistry in Danish finishing pigs at slaughter

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Occurrence and tissue distribution of porcine circovirus type 2 identified by immunohistochemistry in Danish finishing pigs at slaughter

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Summary

Following infection with porcine circovirus type 2 (PCV2) some pigs develop subclinical disease, whereas others express porcine circovirus disease (PCVD), like postweaning multisystemic wasting syndrome (PMWS) and porcine respiratory disease complex (PRDC). In recent years it has been reported that PCV2 infections, PMWS and PCVDs in general, not is observed mainly in the early postweaning period, but now also occur in finishing pigs around 12-19 weeks of age. To asses which role the PCV2 infection plays in older pigs, the occurrence and tissue distribution of PCV2 was examined in Danish finishing pigs at slaughter. Multiple lymph nodes, spleen, lungs and kidneys from 136 pigs with PRDC (case group) and 36 pigs without lung lesions (control group) were examined by immunohistochemistry (IHC) and/or immunofluorescence for PCV2. Additionally, IHC for follicular dendritic cells (FDC) was performed. By IHC 61% of the pigs were positive for PCV2 in 1 or more tissues. Up to 78% of the pigs had mild lymphoid depletion, indistinct lymphoid follicles and/or histiocytic infiltration in the lymph nodes, but the lesions were not associated with the detection of PCV2. Furthermore, no obvious associations were detected between lesions in the lungs or kidneys and detection of PCV2. Three distinct PCV2 IHC staining patterns were seen: a reticular staining pattern of cells with a stellate morphology; staining of isolated non-epithelial cells; and staining of epithelium. The most frequent staining pattern was the reticular, which was seen in the centres of lymphoid follicles, corresponding to the morphology and distribution of FDCs. FDCs trap antigen, like human immunodeficiency virus type 1, as immune complexes along their dendrites. Furthermore, FDCs are intimate with B-lymphocytes and support their proliferation and survival. Based on the detection of PCV2 in relation to FDCs, it should be considered if a reservoir of infective PCV2 is stored on the FDCs or if the virus interacts with the

FDCs causing depletion of B-lymphocytes. In conclusion, PCV2 antigen was associated with FDCs in the tissues of Danish finishing pigs, but the findings were not associated with current PCVDs or PCVD-indicative histopathology. Whether it represents subclinical or persistent infection, or a status after PCV2 infection with innocent storage of antigen, is further to be elucidated.

Keywords: porcine circovirus type 2 (PCV2); porcine circovirus disease (PCVD); follicular dendritic cells (FDC); finishing pigs

Introduction

Porcine circovirus type 2 (PCV2) is a circular single-stranded DNA virus, which is ubiquitous in the swine population and is recognized as the primary causative agent of porcine circovirus disease (PCVD) (Ramamoorthy and Meng, 2008; Segalés *et al.*, 2005a). Several disease complexes are classified as PCVDs, though to date it has only been possible to experimentally reproduce postweaning multisystemic wasting syndrome (PMWS) and PCV2 associated reproductive failure. Thus, these 2 diseases are verified PCVDs, whereas the tentative PCVDs are: porcine respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS), proliferative and necrotizing pneumoniae (PNP), PCV2 associated enteritis, congenital tremor and necrotizing lymphadenitis (Grau-Roma and Segalés, 2007; Jensen *et al.*, 2006; Kim and Chae, 2005; Opriessnig *et al.*, 2007; Stevenson *et al.*, 2001). PCVDs can occur as either systemic disease (e.g. PMWS and PDNS) or relate to specific organs (e.g. PRDC, PCV2 associated reproductive failure and enteritis) (Opriessnig *et al.*, 2007). PMWS is manifested in the postweaning period in pigs of 7-15 weeks of age with most cases occuring from 6-8 weeks of age (Chae, 2004; Harding *et al.*, 1998), whereas PRDC affects older pigs from 14-22 weeks of age (Chae, 2005; Kim *et al.*, 2003). Following PCV2 infection, some pigs develop subclinical disease, i.e. they have no clinical signs together with no or mild lesions and low amounts of PCV2, whereas other pigs express clinical PCVD. The course of infection depends on factors like: susceptibility and immune status of the host, coinfections, and viral genotype (Opriessnig *et al.*, 2007; Segalés, 2002). Since the first case of PMWS was described in 1996 (Harding and Clark, 1997), PMWS has become the most prominent and best characterized PCVD. In order to diagnose PMWS, lymphoid depletion and lymphohistiocytic to granulomatous inflammation must be present in the lymphoid tissues, sometimes accompanied by multinucleated giant cells and presence of basophilic inclusion bodies in histiocytes. Similar lesions may exist in lungs and/or kidneys, intestines, liver and heart. In addition, moderate or massive amounts of PCV2

should be detected in the lesions by immunohistochemistry (IHC) or in situ hybridization (ISH), methods which label the viral protein and DNA, respectively (Chae, 2004; Harding and Halbur, 2002; Opriessnig *et al.*, 2007).

Recently, some reports have claimed that PCV2 infections, PMWS and PCVDs in general, not is observed mainly in the early postweaning period, but now also occur in finishing pigs around 12-19 weeks of age (Carman *et al.*, 2008; D'Allaire *et al.*, 2007; Desrosiers, 2007; Fachinger *et al.*, 2008). Still, however, only few studies of PCVD-indicative histopathology and tissue distribution of PCV2 in finishing pigs have been performed and these studies mainly focus on kidneys and/or a few lymphoid tissues. The results of these studies are furthermore ambiguous as some detected PCV2 in lymphoid organs (Sarli *et al.*, 2006) or kidney lesions (Drolet *et al.*, 2002; Rütten *et al.*, 2006), whereas others did not (Martinez *et al.*, 2006; Quintana *et al.*, 2001).

In the present study, the occurrence and tissue distribution of PCV2 in Danish finishing pigs was examined at slaughter. Multiple lymph nodes, spleen, lungs and kidneys, were investigated by IHC and/or immunofluorescence (IF) for the presence of PCV2 and for identification of the labelled cells, and these findings were compared to PCVD-indicative histopathology. The aim was to asses which role the PCV2 infection plays in older PRDC-affected pigs - focussing on whether the presence of PCV2 antigen in finishing pigs indicates subclinical infection or systemic or organ specific PCVD.

Materials and Methods

Animals and tissues

During 2006 and 2007, lymph nodes (jejunal, tracheobronchial, hepatic and renal), spleen, kidneys and lungs were collected from 172 finishing pigs at two Danish abattoirs. The pigs came from 121 herds. Based on macroscopic and histologic examination of the lungs, reported in another study (Hansen *et al.*, 2009), the animals were divided into a control group without lung lesions (n=36) and a case group with PRDC (n=136). The cases of PRDC were further subdivided as follows: acute bronchopneumonia (n=8); subacute bronchopneumonia (n=20); and chronic bronchopneumonia (n=108). In that study, the lung samples were tested for PCV2 by polymerase chain reaction (PCR), revealing that 133/136 (98%) and 35/36 (97%) of the pigs in the case and control groups, respectively, were positive for PCV2.

Gross Pathology

Lymph nodes, spleen and kidneys were evaluated macroscopically.

Histopathology

The following tissues were examined: jejunal lymph nodes (lnn.) (n=144), tracheobronchial lnn. (n=167), hepatic lnn. (n=88), renal lnn. (n=158), spleen (n=162), kidneys (n=151) and lung tissue from the dorsal surface of the right diapraghma lobe (controls, n=36) and from lesions (cases, n=136). Samples were fixed by immersion in 10% neutral buffered formalin for 24 hours, dehydrated, embedded in paraffin wax and cut in 3-5 µm thick sections. The sections were mounted on conventional glass slides for histochemistry or on SuperFrost®Plus slides (Mensel-gläser, Braunschweig, Germany) for IHC and IF. All sections, except for lungs, were stained by haematoxylin and eosin (HE) (Stevens and Wilson, 1996). The tissue sections were evaluated systematically, e.g. lymph nodes were evaluated with regard to cortex- and medulla-like tissues, lymphoid follicles and sinus systems (Charles, 1996). Lymphoid depletion, i.e. reduced number/density of lymphocytes, was scored semiquantitatively according to Opriessnig *et al.* (2004) as: absent (0); mild lymphoid depletion with loss of overall cellularity (+); moderate lymphoid depletion (++); or severe lymphoid depletion with loss of lymphoid follicle structure (+++).

Immunohistochemistry and immunofluorescence

All tissue samples were examined for presence of PCV2 by IHC, using a non-commercial monoclonal antibody (mAb) (F217) specific for PCV2 capsid antigen (McNeilly *et al.*, 2001). The staining procedure was carried out according to Jensen *et al.* (2006). Additionally, IF for PCV2 was carried out on 15 of the lymph node sections that were positive for PCV2 by IHC. Briefly, sections were deparaffinised, rehydrated and treated with 0.07% protease (Sigma-Aldrich, Brøndby, Denmark), applied for 15 min. at 37°C. Non-specific background staining was blocked by 5% normal swine serum (NSS) (Invitrogen, Taastrup, Denmark) for 10 min. and sections were incubated overnight with the F217 mAb diluted 1:150 in tris-buffered saline (TBS). Subsequently, a 1:350 dilution of Alexa Fluor 488 (Invitrogen, Taastrup, Denmark) in 5% NSS/TBS was applied for 1 hour at 37°C. After washing, the sections were mounted with Fluorescence Mounting Medium (DAKO, Glostrup, Denmark). For detection of fluorescence an AxioImager M1 microscope (Carl Zeiss, Germany) equipped with a 100-W halogen lamp, and filter sets for red and green

fluorescence, was used. Lymph node sections (n=25), representing the observed variation in histopathology, underwent IHC staining procedures aimed at confirming the presence of follicular dendritic cells (FDC) and histiocytes. Detection of FDC was carried out by the use of a 1:75 dilution of a mouse-antihuman mAb, CNA.42 (M 7157, DAKO, Glostrup, Denmark), which labels a 120 kDa protein structure mainly expressed on the cellular membrane (Maeda *et al.*, 2002; Palmieri *et al.*, 2007; Raymond *et al.*, 1997). Briefly, sections were deparaffinised, rehydrated and heated in 0.01M citrate buffer (pH 6.0) in a microwave oven (700 watts) 2 times for 5 min. each, followed by cooling for 15 min. Endogenous peroxidase activity was blocked by 0.5% H_2O_2 for 20 min. Binding of the primary antibody was detected by PowerVision+ HRP kit (Immunovision Technologies, Springdale, AR, USA), applied according to the instructions of the manufacturer, using 3-amino-9-ethylcarbazole (AEC) as chromogen (Kem-En-Tec Diagnostics, Copenhagen, Denmark). Sections were washed in distilled water, counterstained with Mayer's haematoxylin and mounted with glycerol-gelatine. Furthermore, a polyclonal antibody was used to detect S-100, i.e. calcium binding proteins in the cytoplasm of different cells, including FDCs and antigen presenting cells in the lymph nodes (Sarradell *et al.*, 2003; Sato and Dobashi, 1996). IHC for S-100 (Z 0311, DAKO, Glostrup, Denmark) was carried out, slightly modified, according to the description by Soerensen *et al.* (2005). Thus, antigen retrieval was performed as described for the CNA.42 mAb. IHC for lysozym (A 0099, DAKO, Glostrup, Denmark) was slightly modified after a previously described method (Chianini *et al.*, 2003). In general, TBS was used for washing between IHC and IF procedure steps. Furthermore, IHC staining for PCV2 and S-100 was carried out on consecutive lymph node sections (n=10).

Statistics

Data was analysed statistically by Chi-square or Fisher's exact test using SAS version 9.1 (SAS Institute, NC, USA). The statistically significant level set at $P<0.05$.

Results

Gross pathology

No macroscopic lesions were detected in the lymph nodes of animals from the control or case groups. Nodular hyperplasia was seen in the spleen of 1 pig. Kidney lesions were detected in 9% (16/172) of the animals: 5 of these pigs had 1 or more cysts; 11 had white "spots" in medulla and/or cortex, interpreted as chronic interstitial nephritis.

Histopathology

The main histopathological findings of the lymph nodes are summarized in table 1. Mild lymphoid depletion (score +) was detected in 1 or more lymph nodes of 78% (134/172) of the pigs, in total 46% (259/557) of the examined lymph nodes showed signs of depletion (figure 1a). The depletion primarily affected the medulla-like tissues in the subcapsular and peritrabecular areas, and was often regionally distributed, thus affecting some areas of a lymph node, whereas others appeared normal. Another finding in 14% (78/557) of the nodes was indistinct lymphoid follicles, which occurred in 1 or more nodes in 34% (59/172) of the pigs (figure 1b). Furthermore, intergrades of histiocytic infiltration (confirmed by IHC for lysozyme) were seen in the sinus system of the lymph nodes, mainly affecting the subcapsular sinus (figure 1c). This was seen in 75% (129/172) of the pigs in 1 or more lymph nodes; in total 43% (241/557) of the nodes had signs of histiocytic infiltration. Neutrophils in the sinus system were a frequent finding in the tracheobronchial lymph nodes, whereas they were only present in few of the other nodes. Occasional findings were oedema, fibrosis, multinucleated giant cells in the lymphoid follicles and hemosiderin laden macrophages in the sinus system.

Besides nodular hyperplasia in 1 spleen, no significant histologic lesions were observed in the spleens. Chronic interstitial (peritubular, perivascular and periglomerular) lymphohistiocytic nephritis was detected in 95% (143/151) of the kidneys. The relative number of foci present in the kidney sections varied. Furthermore, 1 or more lymphoid nodules were detected in the interstitial tissues of 30% (45/151) of the kidneys (figure 1d).

Statistical analysis of the histopathological findings

When testing for differences between cases and controls in regard to histopathologic findings in the lymph nodes (lymphoid depletion, histiocytic infiltration, indistinct follicles and presence of neutrophils, and multinucleated giant cells), the following 4 associations were observed: in the tracheobronchial nodes an increased number of cases had neutrophils in the sinus system (P=0.002) and more of the controls had histiocytic infiltration (P=0.019). Furthermore, in the renal lymph nodes more controls had indistinct follicles (P=0.042), whereas in the jejunal lymph nodes an increased number of cases had indistinct follicles (P=0.008).

Only in the tracheobronchial nodes (cases and controls pooled) presence of lymphoid depletion was significantly associated with histiocytic infiltration (P<0.001). Otherwise no association was observed between depletion, indistinct follicles and histiocytic infiltration.

The status of depletion (presence or absence) in 1 lymph node was associated to a corresponding finding in minimum 1 of the other nodes for the following nodes (cases and controls pooled): jejunal (P=0.005), tracheobronchial (P=0.002) and renal (P=0.003). This association was also seen in regard to histiocytic infiltration, for the following nodes: jejunal (P=0.011), hepatic (P=0.037) and renal (P=0.007) – but not in regard to the indistinct lymphoid follicles.

PCV2 immunohistochemistry and immunofluorescence

A total of 61% (105/172) of the pigs were positive for PCV2 in 1 or more tissues. Three distinct PCV2 IHC staining patterns were seen: a reticular staining pattern of cells with a stellate morphology; staining of isolated non-epithelial cells; and staining of epithelium (figure 2a-b). The IHC reaction only labelled the cytoplasm of cells, and the staining intensity varied within tissues and between animals. The staining patterns and intensity of the PCV2 IHC and IF reactions, were alike (figure 2c). The different IHC positive tissues and staining patterns of the positive sections (n=242) are presented in table 2. The reticular staining pattern was seen in the centre of lymphoid follicles in lymph nodes and bronchus-associated lymphoid tissue (BALT) structures in the lungs. This was the most common pattern found in the lymph nodes and was seen in 25% (137/557) of the lymph nodes, whereas reticular staining of BALT structures was only seen in 2% (3/172) of the lung sections. The reticular staining was the over all most frequent reaction and was seen in at least 1 section in 42% (73/172) of the animals (table 3). Furthermore staining of isolated cells, morphologically resembling macrophages could be observed in all the different tissues examined, and was the only staining pattern detected in the spleen. The reticular and isolated cell staining patterns could be observed alone or together in lymph nodes. Staining of few epithelium cells was observed in the renal tubules of 7% (10/151) of the kidneys and in a bronchiole of 1% (1/172) of the lungs. The frequency of IHC positive lungs (all staining patterns included) was 14% (19/136) and 17% (6/36), for the cases and controls, respectively. The frequency of IHC positive kidneys (all staining patterns) were 10% (15/151), 7 of these reactions were related to lesions, i.e. infiltration of lymphocytes and histiocytes.

Two pigs that were negative for PCV2 by PCR were labelled by the PCV2 IHC, however not in the lung from where the tissue for PCR was sampled.

Associations between PCV2 immunohistochemistry and pathological findings

No significant difference was observed between cases and controls according to the PCV2 IHC status of the individual tissues (positive or negative) or the status of the individual pigs (i.e. positive pigs having 1 or more positive tissues).

No association was seen between the IHC reaction of the lung and the tracheobronchial lymph nodes (cases and controls pooled). When the PRDC cases were subdivided according to the stage of disease and compared to the IHC results of the lung and/or tracheobronchial lymph node no association was detected. In contrast, the PCV2 IHC status of the pigs was associated to the stage of disease, as most of the acute cases were found to be IHC negative (P=0.009).

In the individual lymph nodes (cases and controls pooled), no associations were observed when comparing the histopathological findings to the IHC status of that lymph node, e.g. in the jejunal lymph node depletion was not associated with IHC positive reaction.

In the kidneys, no association was seen between the PCV2 IHC reaction and presence of gross lesions, interstitial nephritis or lymphoid nodules (cases and controls pooled). Furthermore, when comparing the IHC results of the kidneys to the IHC results of the corresponding lymph node, no significant association was detected (cases and controls pooled).

FDC immunohistochemistry

The staining patterns of CNA.42 and S-100 were similar, i.e. they presented a reticular staining pattern in the centres of lymphoid follicles, corresponding to cells with a stellate morphology, and also stained isolated non-dendritic mononuclear cells in the parafollicular areas (figure 3a-b). The reticular staining patterns resembled that detected by the PCV2 IHC. CNA.42 seemed to label the cellular surface, whereas S-100 stained the cytoplasm and sometimes also the nucleus. The staining intensity of S-100 in the lymph nodes was stronger and more homogeneous compared to CNA.42. The staining intensity of CNA.42 and S-100 tended to be weaker in lymphoid follicles that were positive for PCV2 by IHC, as shown in consecutive tissue sections (figure 3c-d).

Discussion

It is commonly agreed that PCV2 is ubiquitous in the swine population, but existing reports on the prevalence of PCV2 DNA are quite heterogeneous (Opriessnig *et al.*, 2007; Ramamoorthy and Meng, 2008). In the present study 98% of the PRDC cases and 97% of the controls, were positive for PCV2 by PCR (Hansen *et al.*, 2009). Similar results were obtained in recent surveys, where PCV2 DNA was detected in 90% (Chiou *et al.*, 2004), 78% and 73% (Palzer *et al.*, 2008) of pigs with or without pneumonia, whereas surveys from 1996, detected PCV2 DNA in 53% and 19% of healthy slaughtered swine (Liu *et al.*, 2002). These differences might rely on the sensitivity of the detection methods, differences in health status of the animals and/or changes of the epidemiology of PCV2 during time. In the present study 61% of the slaughtered pigs were positive for PCV2 by IHC, which is higher than the results by Sarli *et al.* (2006), who found PCV2 by IHC, in the submaxillary lymph nodes of 34% of regularly slaughtered pigs. In that study the IHC positive lymph nodes were further tested by ISH and the number of positive nodes were thereby reduced to 7%. This difference was interpreted as active infection in the ISH positive animals versus recent exposure to PCV2 in the remaining IHC positive animals. In the present study, all tissues were examined by IHC, whereas PCR only was carried out on lung samples (Hansen *et al.*, 2009). The lung material was selected for lesions compatible with PRDC, a multifactorial respiratory disease entity, which can be caused by multiple pathogens including PCV2 (Allan and McNeilly, 2006; Kim *et al.*, 2003; Thacker, 2006). However, in the present study the detection of PCV2 by IHC could not be directly associated with the lung lesions. Though, it cannot be ruled out that a generalised PCV2 infection have had an effect on the development of PRDC by inducing immunosuppression, an effect that has been ascribed to PCV2 (Segalés *et al.*, 2004), and hereby have prepared the way for other pathogens. PCV2 was detected by IHC in the lungs of both cases and controls with a frequency of 14% and 17%, respectively. But as the IHC detection limit for PCV2 corresponds to 10^8 PCV2 genomes per 500 ng DNA (Brunborg *et al.*, 2004; Segalés *et al.*, 2005b), it was not expected that all PCR positive samples would be positive by IHC. Additionally, the most frequent PCV2 IHC staining pattern of the lungs was labelling of isolated cells, i.e. the chance of including positive cells in a tissue section was lower than the chance of detecting the positive cells/virus by PCR in a larger tissue sample. Thus, these factors might explain the different frequencies of 14-17% and 98% PCV2 positive lungs, detected by IHC and PCR, respectively.

The lymph nodes were found to be the tissue most often positive for PCV2 by IHC. The reticular staining pattern of the PCV2 positive cells in the lymphoid follicles corresponds with the unique morphology and distribution of FDCs, which are present in the centre of primary and secondary lymphoid follicles (Allen and Cyster, 2008). FDCs can also be found in other lymphoid aggregates in various organs (Palmieri *et al.*, 2007; Perez *et al.*, 2001), including BALT structures (Rangel-Moreno *et al.*, 2006). The detection of PCV2 antigen or DNA in the cytoplasm of cells that morphologically resemble porcine FDCs, has been described in healthy slaughtered pigs (Sarli *et al.*, 2006), cases of naturally acquired PCVD (Rosell *et al.*, 2000; Chianini *et al*., 2003) and experimental infection studies including subclinically infected pigs (Krakowka *et al.*, 2002). In the present study, several approaches to perform double labelling of PCV2 and FDCs (by CNA.42) were carried out without success. This depended partly on the heterogenic staining results obtained by CNA.42, which corresponds to the notion that antibodies often label FDCs with heterogenic results (Maeda *et al.*, 2002), maybe because the staining intensity depends on the activation stage of the FDCs; as FDCs become more specialised when the lymph node follicle develops from a primary to a secondary follicle (Allen and Cyster, 2008). In man, the FDC associated protein, identified by the CNA.42 mAb, is expressed during all developmental stages and S-100 is expressed mainly in primary activated FDCs (Kasajima-Akatsuka and Maeda, 2006; Shoji *et al.*, 1998). However, fewer S-100 positive cells were detected after secondary stimulation in rats (Sato and Dobashi, 1996), and in late stages of PMWS (Sarli *et al.*, 2001), which corresponds to the findings in the present study. Thus, this might explain the weaker staining of the FDCs by the CNA.42 mAb and S-100 in the lymphoid follicles that were positive for PCV2. This was demonstrated by IHC staining for PCV2 and S-100 of consecutive lymph node sections, where the same cell type, i.e. FDCs, was labelled by both techniques, showing that the S-100 staining intensity of the PCV2 carrying FDCs was weaker, compared to non-PCV2-associated FDCs (figure 3).

FDCs share the stellate interdigitating morphology of the antigen presenting dendritic cells (DCs), but are otherwise different from these, e.g. they are non-phagocytic and do not express major histocompatibility complex class II (MHCII); however, peptide loaded MHCII molecules can be passively acquired (Allen and Cyster, 2008; Denzer *et al.*, 2000; Gray *et al.*, 1991). It is believed that B-cells or DCs carry antigen to the FDCs, where it is trapped together with antibody and/or complement as immune complexes, which form “beads” (iccosomes) along the dendrites (Allen and Cyster, 2008; Burton *et al.*, 2002; Janeway *et al.*, 2005). Antigens known to be trapped are infective

human immunodeficiency virus type 1 (HIV) (Burton *et al.*, 2002), transmissible spongiform encephalopathy agents (Mabbott and Bruce, 2002), bovine viral diarrhoe virus (Fray *et al.*, 2000), and possible also Epstein-Barr virus (Lindhout *et al.*, 1994; Melot *et al.*, 2004) and bovine herpesvirus 1 (Melot *et al.*, 2004; Winkler *et al.*, 2000). HIV can be retained on the surface of FDCs for months to years, without the cells being infected, thus FDCs protect the virus from degradation and act as a reservoir for HIV (Burton *et al.*, 2002; Thacker *et al.*, 2009). Persistence of PCV2 viremia, lasting for up to 12 weeks, has been detected by PCR in subclinically infected pigs of 21-25 weeks of age in field studies (Carasova *et al.*, 2007; Larochelle *et al.*, 2003; Sibila *et al.*, 2004), findings, which are supported experimentally (Bolin *et al.*, 2001; Darwich *et al.*, 2008). Previously it has been proposed that the lymph nodes, near the site of infection, may be important in maintaining persistence of PCV2 (Yu *et al.*, 2007). Though HIV and PCV2 are different viruses, the detection of PCV2 in relation to FDCs might indicate that a similar PCV2 reservoir is maintained by the FDCs. Thus, further examinations should be carried out to determine if the viral antigen associated to FDCs is intact and infective, i.e. represents persistent or subclinical infection, or is an accumulation of viral antigen fragments, thus representing a status after PCV2 infection.

FDCs are in close contact with B-lymphocytes in the lymphoid follicles and are believed to have important functions in the development of naïve B-cells into activated B-cells, e.g. they stimulate proliferation and prevent apoptosis of the B-cells (Allen and Cyster, 2008; Park and Choi, 2005). In experimental PCV2 infection studies (Yu *et al.*, 2007) and natural cases of PMWS (Sarli *et al.*, 2001; Segalés *et al.*, 2004), loss of B-cells in lymphoid follicles is an early sign of infection. The reduction in number of B-lymphocytes has been ascribed to different mechanisms, like apoptosis (Shibahara *et al.*, 2000) or reduced proliferation (Mandrioli *et al.*, 2004; Resendes *et al.*, 2004). Based on the findings in the present study it could be considered, if PCV2 interacts with FDCs in a way that interferes with the maturation and survival of B-cells? Thus, the interactions between B-cells, FDCs and PCV2 in regard to lymphoid depletion should be examined further.

Mild lymphoid depletion, histiocytic infiltration and indistinct follicles were detected in one or more lymph nodes of 78%, 75% and 34% of the pigs, respectively. These histopathologic findings are characteristic of PMWS, but in this study the lesions were not as severe as in most cases of PMWS (Chae, 2004; Chianini *et al.*, 2003; Rosell *et al.*, 1999), and they were not associated with the presence of PCV2. In general, if depletion or histiocytic infiltration was present, this was found

in several lymph nodes from the same pig. Lymphoid depletion and histiocytic infiltration have been reported in 10-14 weeks old pigs subclinically infected with PCV2 (Nielsen *et al.*, 2008; Quintana *et al.*, 2001), however these reactions are not specific for PCV2 infections (Bodewadt-Radzun *et al.*, 1990; Charles, 1996; Jaffe, 1988; Woda and Sullivan, 1993). Thus, the lesions found in the lymph nodes of the finishing pigs do not seem to be associated with PCVD.

When the kidneys are affected in PMWS, PCV2 is often detected in the renal tubular epithelium (Sarli *et al.*, 2008), corresponding with the present study, where PCV2 was detected in the tubular epithelium of 7% of the pigs. The antigen was neither significantly associated with macroscopically interstitial nephritis nor with histological lesions, whereas Rütten *et al.* (2006) detected PCV2 in the tubular epithelium of 38% of slaughtered pigs with gross lesions of chronic interstitial nephritis. Other divergent reports on the association between presence of PCV2 and "white spotted kidneys" in slaughtered pigs exist, where some detected an association (Drolet *et al.*, 2002), whereas others did not (Martinez *et al.*, 2006). As a curiosity, lymphoid nodules were detected in 30% of the kidneys and similar nodules have been described in the kidneys of finishing pigs in surveys of PCVD (Drolet *et al.*, 2002; Martinez *et al.*, 2006; Rütten *et al.*, 2006). In these surveys PCV2 and lymphoid nodules were not believed to be associated, which is in agreement with the results of the present study. Thus these lesions are probably a non-specific response to chronic inflammation.

PCV2 is believed to be transmitted mainly through the oronasal route (Ramamoorthy and Meng, 2008) and the virus is excreted in several body fluids, i.e. respiratory and oral secretions, urine, semen and colostrum (Park *et al.*, 2009; Schmoll *et al.*, 2008; Segalés *et al.*, 2005b; Shibata *et al.*, 2006). The excretion of PCV2 in urine and the finding of PCV2 antigen mainly in the tubular epithelium of the kidneys, in the present and other studies (Rütten *et al.*, 2006; Sarli *et al.*, 2008), gives evidence for dissemination of PCV2 from finishing pigs to the environment, which could result in horizontal transmission to susceptible pigs.

In conclusion, PCV2 antigen was detected by IHC in the tissues of 61% of Danish finishing pigs, but the findings were not associated with current PCVD or PCVD-indicative histopathology. The PCV2 IHC reaction was mainly associated with lymphoid follicles in the lymph nodes, where FDCs were labelled, but whether it represents subclinical or persistent infection, or a status after PCV2

infection with innocent storage of antigen, is further to be elucidated. Moreover, interactions between B-cells, FDCs and PCV2 should be examined further.

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References

Allen, C. D. and Cyster, J. G. (2008). Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Seminars in Immunology*, **20**, 14-25.

Allan, G. and McNeilly, F. (2006). PMWS/PCVD: Diagnose, disease, and control: What do we know? In: *Proceedings of the 19th International Pig Veterinary Society Congress*, Copenhagen, Denmark, pp. 1-9.

Bodewadt-Radzun, S., Wacker, H. H., Radzun, H. J. and Parwaresch, M. R. (1990). Phenotypic characterization of macrophages in non-neoplastic lymph nodes. *Current Topics in Pathology*, **84 (Pt 1)**, 247-264.

Bolin, S. R., Stoffregen, W. C., Nayar, G. P. and Hamel, A. L. (2001). Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-deprived piglets with type 2 porcine circovirus. *Journal of Veterinary Diagnstic Investigation,* **13**, 185-194.

Brunborg, I. M., Moldal, T. and Jonassen, C. M. (2004). Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. *Journal of Virological Methods*, **122**, 171-178.

Burton, G. F., Keele, B. F., Estes, J. D., Thacker, T. C. and Gartner, S. (2002). Follicular dendritic cell contributions to HIV pathogenesis. *Seminars in Immunology*, **14**, 275-284.

Carasova, P., Celer, V., Takacova, K., Trundova, M., Molinkova, D., Lobova, D. and Smola, J. (2007). The levels of PCV2 specific antibodies and viremia in pigs. *Research in Veterinary Science*, **83**, 274-278.

Carman, S., Cai, H. Y., DeLay, J., Youssef, S. A., McEwen, B. J., Gagnon, C. A., Tremblay, D., Hazlett, M., Lusis, P., Fairles, J., Alexander, H. S. and van, D. T. (2008). The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease—2004-2006. *Canadian Journal of Veterinary Research*, **72**, 259-268.

Chae, C. (2005). A review of porcine circovirus 2-associated syndromes and diseases. *Veterinary Journal*, **169**, 326-336.

Chae, C. (2004). Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. *Veterinary Journal*, **168**, 41-49.

Charles, J. A. (1996). Lymph Nodes and Thymus. In: *Pathology of the Pig. A Diagnostic Guide*, L. D. Sims and J. R. W. Glastonbury, Eds., The Pig Research and Development Corporation and Agriculture Victoria, Victoria, pp. 185-210.

Chianini, F., Majo, N., Segalés, J., Dominguez, J. and Domingo, M. (2003). Immunohistochemical characterisation of PCV2 associate lesions in lymphoid and non-lymphoid tissues of pigs with natural postweaning multisystemic wasting syndrome (PMWS). *Veterinary Immunology and Immunopathology*, **94**, 63-75.

Chiou, M. T., Lin, C. N., Chuang, M. S., Chiang, H. C. and Tsai, C. P. (2004). Etiological and epidemiological survey of PRDC associated pathogens in Taiwan. In: *Proceedings of the 18th International Pig Veterinary Society Congress*, Hamburg, Germany, p. 191.

D'Allaire, S., Moore, C. and Cote, G. (2007). A survey on finishing pig mortality associated with porcine circovirus diseases in Quebec. *Canadian Veteterinary Journal*, **48**, 145-146.

Darwich, L., Segalés, J., Resendes, A., Balasch, M., Plana-Duran, J. and Mateu, E. (2008). Transient correlation between viremia levels and IL-10 expression in pigs subclinically infected with porcine circovirus type 2 (PCV2). *Research in Veterinary Science*, **84**, 194-198.

Denzer, K., van, E. M., Kleijmeer, M. J., Jakobson, E., de, G. C. and Geuze, H. J. (2000). Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. *Journal of Immunolgy*, **165**, 1259-1265.

Desrosiers, R. (2007). Overview of PCVD - The disease in Eastern Canada and US vs. Europe. In: *Proceedings of the 2007 Banff Pork Seminar*, Alberta, Canada, pp. 35-48.

Drolet, R., D'Allaire, S., Larochelle, R., Magar, R., Ribotta, M. and Higgins, R. (2002). Infectious agents identified in pigs with multifocal interstitial nephritis at slaughter. *The Veterinary Record*, **150**, 139-143.

Fachinger, V., Bischoff, R., Jedidia, S. B., Saalmuller, A. and Elbers, K. (2008). The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. *Vaccine*, **26**, 1488-1499.

Fray, M. D., Supple, E. A., Morrison, W. I. and Charleston, B. (2000). Germinal centre localization of bovine viral diarrhoea virus in persistently infected animals. *The Journal of general virology*, **81**, 1669-1673.

Grau-Roma, L. and Segalés, J. (2007). Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. *Veteterinary Microbiolology*, **119**, 144-151.

Gray, D., Kosco, M. and Stockinger, B. (1991). Novel pathways of antigen presentation for the maintenance of memory. *International Immunology*, **3**, 141-148.

Hansen, M. S., Pors, S. E., Jensen, H. E., Bille-Hansen, V., Bisgaard, M., Flachs, E. M., & Nielsen, O. L. (2009). Porcine respiratory disease complex in Danish finishing pigs - update on pathology and pathogens. *Journal of Comparative Pathology*, submitted.

Harding, J. C. and Halbur, P. G. (2002). PMWS or a Group of PCV2-Associated Syndromes: Ever-Growing Concerns. In: *PMWS and PCV2 Diseases, Beyond the Debate, Keynotes on the Merial Symposium and Brief Epidemiological Updates.* The 17th International Pig Veterinary Society Congress, Ames, Iowa, USA, pp. 19-31.

Harding, J. C. S. and Clark, E. G. (1997). Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). *The Journal of Swine Health and Production*, **5**, 201-203.

Harding, J. C. S., Clark, E. G., Strokappe, J. H., Willson, P. I. and Ellis, J. A. (1998). Postweaning multisystemic wasting syndrome: Epidemiology and clinical presentation. *The Journal of Swine Health and Production*, **6**, 249-254.

Jaffe, E. S. (1988). Histiocytoses of lymph nodes: biology and differential diagnosis. *Seminars in Diagnostic Pathology*, **5**, 376-390.

Janeway, C. A. Jr., Travers, P., Walport, M. and Shlomchik, M. J. (2005). *Immunobiology: the immune system in health and disease*, 6 Edit., Garland Science Publishing, New York.

Jensen, T. K., Vigre, H., Svensmark, B. and Bille-Hansen, V. (2006). Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by Lawsonia intracellularis. *Journal of Comparative Pathology*, **135**, 176-182.

Kasajima-Akatsuka, N. and Maeda, K. (2006). Development, maturation and subsequent activation of follicular dendritic cells (FDC): immunohistochemical observation of human fetal and adult lymph nodes. *Histochemistry and Cell Biology*, **126**, 261-273.

Kim, J. and Chae, C. (2005). Necrotising lymphadenitis associated with porcine circovirus type 2 in pigs. *The Veterinary Record*, **156**, 177-178.

Kim, J., Chung, H. K. and Chae, C. (2003). Association of porcine circovirus 2 with porcine respiratory disease complex. *Veterinary Journal*, **166**, 251-256.

Krakowka, S., Ellis, J. A., McNeilly, F., Gilpin, D., Meehan, B., McCullough, K. and Allan, G. (2002). Immunologic features of porcine circovirus type 2 infection. *Viral Immunolgy*, **15**, 567-582.

Larochelle, R., Magar, R. and D'Allaire, S. (2003). Comparative serologic and virologic study of commercial swine herds with and without postweaning multisystemic wasting syndrome. *Canadian Journal of Veterinary Research*, **67**, 114-120.

Lindhout, E., Lakeman, A., Mevissen, M. L. and de, G. C. (1994). Functionally active Epstein-Barr virus-transformed follicular dendritic cell-like cell lines. *Journal of Experimental Medicine*, **179**, 1173-1184.

Liu, Q., Wang, L., Willson, P., O'Connor, B., Keenliside, J., Chirino-Trejo, M., Melendez, R. and Babiuk, L. (2002). Seroprevalence of porcine circovirus type 2 in swine populations in Canada and Costa Rica. *Canadian Journal of Veterinary Research*, **66**, 225-231.

Mabbott, N. A. and Bruce, M. E. (2002). Follicular dendritic cells as targets for intervention in transmissible spongiform encephalopathies. *Seminars in Immunology*, **14**, 285-293.

Maeda, K., Matsuda, M., Suzuki, H. and Saitoh, H. A. (2002). Immunohistochemical recognition of human follicular dendritic cells (FDCs) in routinely processed paraffin sections. *Journal of Histochemistry and Cytochemistry*, **50**, 1475-1486.

Mandrioli, L., Sarli, G., Panarese, S., Baldoni, S. and Marcato, P. S. (2004). Apoptosis and proliferative activity in lymph node reaction in postweaning multisystemic wasting syndrome (PMWS). *Veterinary Immunology and Immunopathology*, **97**, 25-37.

Martinez, J., Segalés, J., Aduriz, G., Atxaerandio, R., Jaro, P., Ortega, J., Peris, B. and Corpa, J. M. (2006). Pathological and aetiological studies of multifocal interstitial nephritis in wasted pigs at slaughter. *Research in Veterinary Science*, **81**, 92-98.

McNeilly, F., McNair, I., Mackie, D. P., Meehan, B. M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S. and Allan, G. M. (2001). Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. *Archives of Virology*, **146**, 909-922.

Melot, F., Defaweux, V., Jolois, O., Collard, A., Robert, B., Heinen, E. and Antoine, N. (2004). FDC-B1: a new monoclonal antibody directed against bovine follicular dendritic cells. *Veterinary Immunology and Immunopathology*, **97**, 1-9.

Nielsen, E. O., Enoe, C., Jorsal, S. E., Barfod, K., Svensmark, B., Bille-Hansen, V., Vigre, H., Botner, A. and Baekbo, P. (2008). Postweaning multisystemic wasting syndrome in Danish pig herds: productivity, clinical signs and pathology. *The Veterinary Record*, **162**, 505-508.

Opriessnig, T., Meng, X. J. and Halbur, P. G. (2007). Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *Journal of Veterinary Diagnostic Investigation*, **19**, 591-615.

Opriessnig, T., Thacker, E. L., Yu, S., Fenaux, M., Meng, X. J. and Halbur, P. G. (2004). Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. *Veterinary Pathology,* **41**, 624-640.

Palmieri, C., Brunetti, M. and Della, S. L. (2007). Immunohistochemical characterization of Kisselev nodules (ectopic lymphoid follicles) in wild boar (Sus scrofa L.). *Research in Veterinary Science*, **83**, 109-115.

Palzer, A., Ritzmann, M., Wolf, G. and Heinritzi, K. (2008). Associations between pathogens in healthy pigs and pigs with pneumonia. *The Veterinary Record*, **162**, 267-271.

Park, C. S. and Choi, Y. S. (2005). How do follicular dendritic cells interact intimately with B cells in the germinal centre? *Immunology*, **114**, 2-10.

Park, J. S., Ha, Y., Kwon, B., Cho, K. D., Lee, B. H. and Chae, C. (2009). Detection of Porcine Circovirus 2 in Mammary and Other Tissues from Experimentally Infected Sows. *Journal of Comparative Pathology,* **140**, 208-211.

Perez, J., Garcia, P. M., Mozos, E., Bautista, M. J. and Carrasco, L. (2001). Immunohistochemical characterization of hepatic lesions associated with migrating larvae of Ascaris suum in pigs. *Journal of Comparative Pathology*, **124**, 200-206.

Quintana, J., Segalés, J., Rosell, C., Calsamiglia, M., Rodriguez-Arrioja, G. M., Chianini, F., Folch, J. M., Maldonado, J., Canal, M., Plana-Duran, J. and Domingo, M. (2001). Clinical and pathological observations on pigs with postweaning multisystemic wasting syndrome. *The Veterinary Record*, **149**, 357-361.

Ramamoorthy, S. and Meng, X. (2008). Porcine circoviruses: a minuscule yet mammoth paradox. *Animal Health Research Reviews*, **doi:10.1017/S1466252308001461**.

Rangel-Moreno, J., Hartson, L., Navarro, C., Gaxiola, M., Selman, M. and Randall, T. D. (2006). Inducible bronchus-associated lymphoid tissue (iBALT) in patients with pulmonary complications of rheumatoid arthritis. *The Journal of Clinical Investigation,* **116**, 3183-3194.

Raymond, I., Al, S. T., Tkaczuk, J., Chittal, S. and Delsol, G. (1997). CNA.42, a new monoclonal antibody directed against a fixative-resistant antigen of follicular dendritic reticulum cells. *The American Journal of Pathology,* **151**, 1577-1585.

Resendes, A. R., Majo, N., Segalés, J., Mateu, E., Calsamiglia, M. and Domingo, M. (2004). Apoptosis in lymphoid organs of pigs naturally infected by porcine circovirus type 2. *The Journal of general virology*, **85**, 2837-2844.

Rosell, C., Segalés, J., Ramos-Vara, J. A., Folch, J. M., Rodriguez-Arrioja, G. M., Duran, C. O., Balasch, M., Plana-Duran, J. and Domingo, M. (2000). Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. *The Veterinary Record*, **146**, 40-43.

Rosell, C., Segalés, J., Plana-Duran, J., Balasch, M., Rodriguez-Arrioja, G. M., Kennedy, S., Allan, G. M., McNeilly, F., Latimer, K. S. and Domingo, M. (1999). Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. *Journal of Comparative Pathology*, **120**, 59-78.

Rütten, M., Stephan, R., Pospischil, A. and Sydler, T. (2006). Porcine circovirus type 2 infections in healthy pigs at slaughter. In: *The 24th meeting of the European Society of Veterinary Pathology*, Edinburgh, Scotland, pp. 108-109.

Sarli, G., Mandrioli, L., Panarese, S., Brunetti, B., Segalés, J., Dominguez, J. and Marcato, P. S. (2008). Characterization of interstitial nephritis in pigs with naturally occurring postweaning multisystemic wasting syndrome. *Veterinary Pathology*, **45**, 12-18.

Sarli, G., Bacci, B., Brunetti, B., Panarese, S., Mandroli, L., Ostanello, F., Caprioli, A., Cerati, C. and Marcato, P. S. (2006). PCV2 detection in 170 submaxillary lymphnodes of regularly slaughtered pigs in Northern Italy. In: *Proceedings of the 19th International Pig Veterinary Society Congress*, Copenhagen, Denmark, p. 85.

Sarli, G., Mandrioli, L., Laurenti, M., Sidoli, L., Cerati, C., Rolla, G. and Marcato, P. S. (2001). Immunohistochemical characterisation of the lymph node reaction in pig post-weaning multisystemic wasting syndrome (PMWS). *Veterinary Immunolgy and Immunopathology*, **83**, 53-67.

Sarradell, J., Andrada, M., Ramirez, A. S., Fernandez, A., Gomez-Villamandos, J. C., Jover, A., Lorenzo, H., Herraez, P. and Rodriguez, F. (2003). A morphologic and immunohistochemical study of the bronchus-associated lymphoid tissue of pigs naturally infected with Mycoplasma hyopneumoniae. *Veterinary Pathology*, **40**, 395-404.

Sato, H. and Dobashi, M. (1996). Ultrastructural localization of S-100 protein in rat popliteal lymph nodes, and very slight proliferative activity of follicular dendritic cells. *Histology and Histopathology*, **11**, 703-709.

Schmoll, F., Lang, C., Steinrigl, A. S., Schulze, K. and Kauffold, J. (2008). Prevalence of PCV2 in Austrian and German boars and semen used for artificial insemination. *Theriogenology*, **69**, 814-821.

Segalés, J., Allan, G. M. and Domingo, M. (2005a). Porcine circovirus diseases. *Animal Health Research Reviews*, **6**, 119-142.

Segalés, J., Calsamiglia, M., Olvera, A., Sibila, M., Badiella, L. and 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). *Veterinary Microbiology*, **111**, 223-229.

Segalés, J., Domingo, M., Chianini, F., Majo, N., Dominguez, J., Darwich, L. and Mateu, E. (2004). Immunosuppression in postweaning multisystemic wasting syndrome affected pigs. *Veterinary Microbiology*, **98**, 151-158.

Segalés, J. (2002). Update on postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome diagnostics. *The Journal of Swine Health and Production*, **10**, 277-281.

Shibahara, T., Sato, K., Ishikawa, Y. and Kadota, K. (2000). Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. *Journal of Veterinary Medical Science*, **62**, 1125-1131.

Shibata, I., Okuda, Y., Kitajima, K. and Asai, T. (2006). Shedding of porcine circovirus into colostrum of sows. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health*, **53**, 278-280.

Shoji, J., Inada, N., Saito, K., Takaura, N., Iwasaki, Y. and Sawa, M. (1998). Immunohistochemical study on follicular dendritic cell of conjunctiva-associated lymphoid tissue. *Japanese Journal of Ophthalmology*, **42**, 1-7.

Sibila, M., Calsamiglia, M., Segalés, J., Blanchard, P., Badiella, L., Le, D. M., Jestin, A. and Domingo, 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. *American Journal of Veterinary Research*, **65**, 88-92.

Soerensen, C. M., Holmskov, U., Aalbaek, B., Boye, M., Heegaard, P. M. and Nielsen, O. L. (2005). Pulmonary infections in swine induce altered porcine surfactant protein D expression and localization to dendritic cells in bronchial-associated lymphoid tissue. *Immunology*, **115**, 526-535.

Stevens, A. and Wilson, I. G. (1996). The haematoxylins and eosin. In: *Theory and practice of Histological Techniques*, 4 Edit., J. D. Bancroft and A. Stevens, Eds., Churchill Livingstone, New York, pp. 99-112.

Stevenson, G. W., Kiupel, M., Mittal, S. K., Choi, J., Latimer, K. S. and Kanitz, C. L. (2001). Tissue distribution and genetic typing of porcine circoviruses in pigs with naturally occurring congenital tremors. *Journal of Veterinary Diagnostic Investigation*, **13**, 57-62.

Thacker, E. L. (2006). Mycoplasmal Diseases. In: *Diseases of Swine*, 9 Edit., B. E. Straw, J. J. Zimmerman, S. D'Allaire and D. J. Taylor, Eds., Blackwell Publishing, Oxford, pp. 701-717.

Thacker, T. C., Zhou, X., Estes, J. D., Jiang, Y., Keele, B. F., Elton, T. S. and Burton, G. F. (2009). Follicular dendritic cells and human immunodeficiency virus type 1 transcription in CD4+ T cells. *Journal of Virology*, **83**, 150-158.

Winkler, M. T., Doster, A. and Jones, C. (2000). Persistence and reactivation of bovine herpesvirus 1 in the tonsils of latently infected calves. *Journal of Virologly*, **74**, 5337-5346.

Woda, B. A. and Sullivan, J. L. (1993). Reactive histiocytic disorders. *American Journal of Clinical Pathology*, **99**, 459-463.

Yu, S., Opriessnig, T., Kitikoon, P., Nilubol, D., Halbur, P. G. and Thacker, E. (2007). Porcine circovirus type 2 (PCV2) distribution and replication in tissues and immune cells in early infected pigs. *Veterinary Immunolgy and Immunopathology*, **115**, 261-272.

Tables

Table 1
Main histopathological findings in the lymph nodes of slaughtered finishing pigs

	Lymph nodes							
	Jejunal		Tracheobronchial		Hepatic		Renal	
	Cases* (n=109)	Controls* (n=35)	Cases* (n=132)	Controls* (n=35)	Cases* (n=66)	Controls* (n=22)	Cases* (n=125)	Controls* (n=33)
Lymphoid depletion†	46 (42%)	10 (29%)	66 (50%)	24 (69%)	40 (61%)	11 (50%)	55 (44%)	11 (33%)
Histiocytic infiltration	53 (49%)	17 (49%)	45 (34%)	20 (57%)	29 (44%)	13 (59%)	51 (41%)	17 (52%)
Indistinct follicles	19 (17%)	0	14 (11%)	2 (6%)	23 (35%)	5 (23%)	15 (12%)	0
Neutrophils in sinus system	2 (2%)	1 (3%)	34 (26%)	1 (3%)	3 (5%)	1 (5%)	2 (2%)	0
Presence of multinucleate giant cells	3 (3%)	0	1 (1%)	1 (1%)	4 (6%)	3 (14%)	3 (2%)	0

* Cases of porcine respiratory disease complex and controls without lung lesions
† Only mild lymphoid depletion (grade +) with loss of overall cellularity was detected

Table 2
Porcine circovvirus type 2 (PCV2) immunohistochemistry (IHC) positive sections (n=242) from slaughtered finishing pigs, distributed according to tissues and staining pattern

	IHC staining pattern		
Tissue	Reticular*	Isolated cells†	Epithelium
Lymph nodes			
Jejunal			
Cases‡ (n=109)	22 (20%)	9 (8%)	-§
Controls‡ (n=35)	8 (23%)	3 (9%)	-
Tracheobronchial			
Cases‡ (n=132)	29 (23%)	10 (8%)	-
Controls‡ (n=35)	12 (34%)	1 (3%)	-
Hepatic			
Cases‡ (n=66)	22 (33%)	2 (3%)	-
Controls‡ (n=22)	5 (23%)	-	-
Renal			
Cases‡ (n=125)	27 (22%)	11 (9%)	-
Controls‡ (n=33)	12 (36%)	1 (3%)	-
Spleen			
Cases‡ (n=127)	-	21 (17%)	-
Controls‡ (n=35)	-	7 (20%)	-
Kidney			
Cases‡ (n=118)	-	4 (3%)	9 (8%)
Controls‡ (n=33)	-	1 (3%)	1 (3%)
Lung			
Cases‡ (n=136)	1 (1%)	17 (13%)	1 (1%)
Controls‡ (n=36)	3 (8%)	3 (8%)	-
Total	141 (58%)	90 (37%)	11 (5%)

* Reticular staining of PCV2 antigen in the centre of lymphoid follicles in lymph nodes or the bronchus-associated lymphoid tissue (BALT) in the lung
† Isolated non-epithelial cells
‡ Cases of porcine respiratory disease complex and controls without lung lesions
§ IHC stainings negative

Table 3
Number of slaughtered finishing pigs (n=105) positive for porcine circovirus type 2 by immunohistochemistry (IHC), distributed according to staining pattern and number of positive tissues per pig

	Number of IHC positive tissues per pig							
IHC staining pattern	1	2	3	4	5	6	7	Total
Reticular*	20 (19%)	18 (17%)	19 (18%)	10 (9%)	3 (3%)	2 (2%)	1 (1%)	73 (70%)
Only isolated cells†	14 (13%)	9 (9%)	2 (2%)	2 (2%)	1 (1%)	0	0	28 (27%)
Only epithelium‡	4 (4%)	0	0	0	0	0	0	4 (4%)
Total	38 (36%)	27 (26%)	21 (20%)	12 (11%)	4 (4%)	2 (2%)	1 (1%)	105 (100%)

* Reticular staining pattern was observed in minimum 1 tissue section in these pigs, i.e. in lymphoid follicles of the lymph nodes or bronchus-associated lymphoid tissue (BALT) in the lungs. Isolated non-epithelial cells or epithelium were also positive in some of the sections of these pigs, e.g. in the spleen or the kidneys
† Only isolated non-epithelial cells were stained, i.e. reticular staining or staining of epithelium was not observed in any of the sections from these pigs
‡ Only epithelium was stained, i.e. reticular staining or staining of isolated non-epithelial cells was not observed in any of the sections from these pigs

Figure legends

Fig. 1. Histology of tissues from slaughtered finishing pigs. (a) Lymph node section showing mild lymphoid depletion (grade +) with loss of overall cellularity. Hematoxylin and eosin stain (HE). Bar, 200 μm. (b) Lymph node section showing indistinct follicles (marked by f). HE. Bar, 200 μm. (c) Immunohistochemistry for lysozyme of a lymph node section with infiltration of histiocytes in the sinus system. The lymph node capsule is marked by an asterisk. Counterstained with hematoxylin. Bar, 85 μm. (d) Kidney section with presence of several lymphoid nodules. HE. Bar, 85 μm.

Fig. 2. Immunohistochemistry (IHC) and immunofluorescence (IF) for porcine circovirus type 2. (a) ICH of lymph node section, showing the reticular staining pattern, which was associated to cells with a stellate morphology in the centre of follicles. Counterstained with hematoxylin. Bar, 20 μm. (b) IHC of kidney section with positive tubular epithelium (arrows) and associated lymphohistiocytic infiltration. Counterstained with hematoxylin. Bar, 20 μm. (c) Section of a lymph node stained by IF. The staining is reticular and was associated to cells with a stellate morphology in the centre of follicles, i.e. resembling that of the PCV2 IHC. Bar, 20 μm.

Fig. 3. Lymph node sections stained by immunohistochemistry (IHC) for follicular dendritic cells (FDCs) or porcine circovirus type 2 (PCV2). Counterstained with hematoxylin. (a) IHC with CNA.42 showing the reticular staining pattern of FDCs. Bar, 85 μm. Insert: close up on FDCs, showing the spotted labelling and stellate morphology. Bar, 13 μm. (b) IHC for S-100 showing the reticular homogeneous staining pattern of FDCs. S-100 labels the cytoplasm and sometimes also the nucleus. Bar, 85 μm. Insert: close up on S-100 positive FDCs. Bar, 20 μm. (c) and (d) IHC of consecutive tissue sections for PCV2 and S-100, respectively, showing similar staining of follicle centres. S-100 also stained isolated non-dendritic mononuclear cells in the paracortical tissues of the lymph node. Bar, 40 μm.

Figures

Figure 1

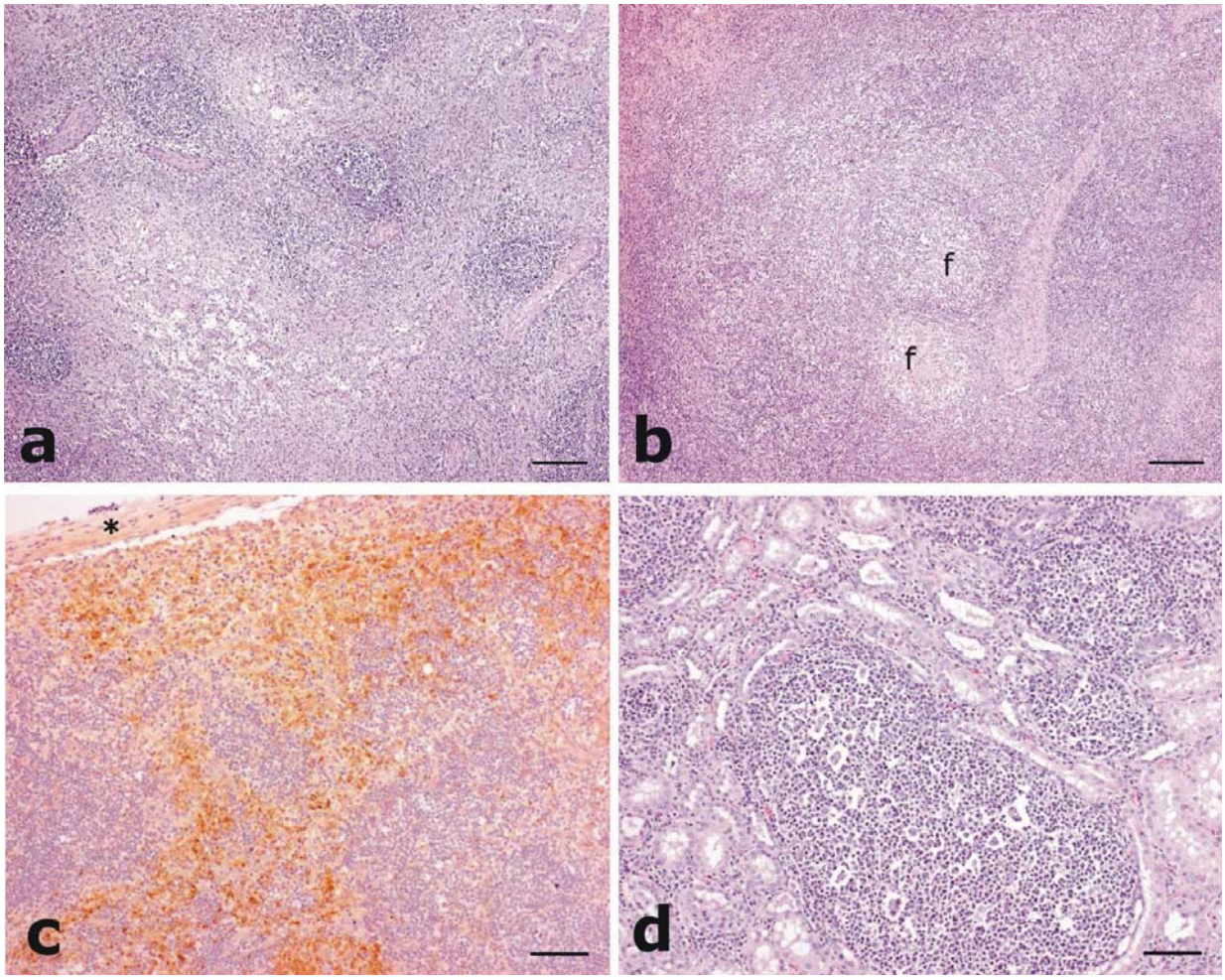


Figure 2

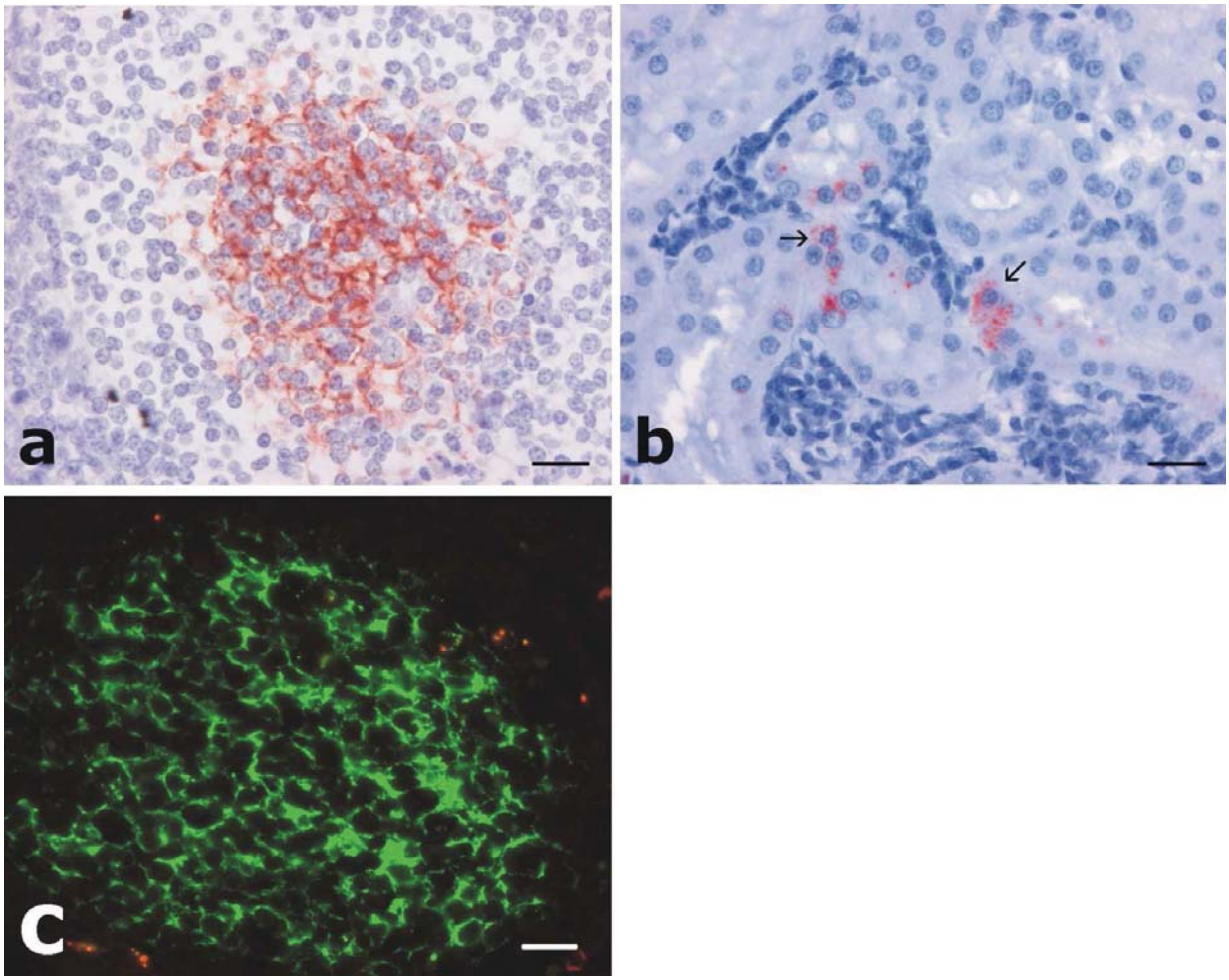
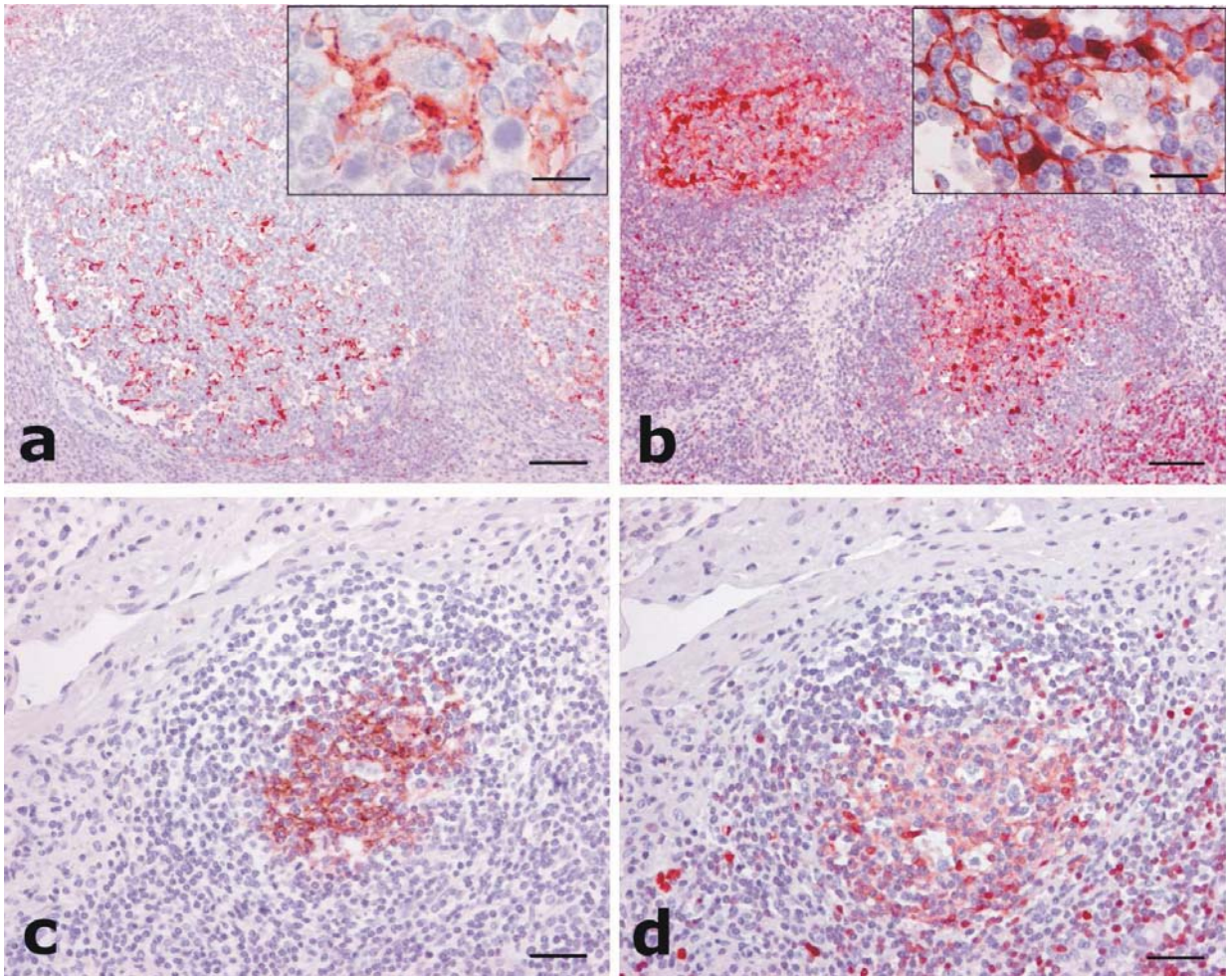


Figure 3



Manuscript III

Time related diagnostic parameters of porcine circovirus type 2 associated reproductive failure in a Danish herd

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Time related diagnostic parameters of porcine circovirus type 2 associated reproductive failure in a Danish herd

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Abstract

During a 2 month period a newly repopulated Danish pig herd experienced an increase in numbers of stillborn and mummies, caused by porcine circovirus type 2 (PCV2) associated reproductive failure. Based on recordings of data over time, the progression of foetal infection was studied and the diagnostic value of different techniques was evaluated. Foetuses, cases (n=38) and controls (n=13), were examined by immunohistochemistry (IHC) and real-time polymerase chain reaction (PCR) for detection of PCV2; and total immunoglobulin G (IgG) was measured. PCV2 replication was detected by in situ hybridization (ISH). PCV2 IHC was positive in 37% of the case foetuses and replication was detected in 43% of the IHC positive hearts. All IHC positive foetuses were delivered during a 9 days period early in the outbreak. On basis of the results obtained by IHC and PCR, the foetuses were divided into 3 categories: PCV2 negative (DNA load below cut-off); suspected positive (DNA load 10^4-10^7); and PCV2 positive (DNA load $>10^7$). All control foetuses and IHC positive foetuses were included in the negative and positive groups, respectively. Ten case foetuses had elevated IgG levels, which was not associated with the IHC or PCR results. Based on the clustering of the IHC positive foetuses, it is suggested that IHC only is suited for diagnosing acute stages of reproductive failure, whereas quantitative PCR can be used as a sensitive diagnostic method within a wider time span. Furthermore, it seems that IgG measurements are not very reliable as indication of intrauterine infection with PCV2.

Keywords: immunohistochemistry (IHC); immunoglobulin G (IgG); porcine circovirus type 2 (PCV2) associated reproductive failure; real-time polymerase chain reaction (PCR)

Introduction

Porcine circovirus type 2 (PCV2) is regarded as the primary causative agent of porcine circovirus diseases (PCVDs), like postweaning multisystemic wasting syndrome (PMWS), porcine respiratory disease complex (PRDC) and reproductive disorders (Opriessnig et al., 2007; Segalés et al., 2005). PCV2 associated reproductive failure can be reproduced experimentally and these studies have shown that porcine embryos and foetuses are susceptible to PCV2 infection (Johnson et al., 2002; Mateusen et al., 2007; Mateusen et al., 2004), and that intrauterine spread (Pensaert et al., 2004) and vertical transmission can occur (Nielsen et al., 2004; Park et al., 2005; Rose et al., 2007). The first case description of PCV2 associated reproductive failure was in 1999 (West et al., 1999) and it has been confirmed by subsequent similar incidents, mainly from newly established production facilities with first parity gilts (Brunborg et al., 2007; Josephson and Charbonneau, 2001; Ladekjaer-Mikkelsen et al., 2001; O'Connor et al., 2001; Pittman, 2008). According to Segalés et al. (2006) the following criteria must be fulfilled in order to diagnose reproductive problems as PCV2 associated: an increase in the number of abortions and/or stillbirths and mummified foetuses, together with fibrotic and/or necrotizing myocardial lesions in the foetuses and detection of PCV2 in the lesions. The standard methods for detection of PCV2 is by immunohistochemistry (IHC) and in situ hybridization (ISH) (Sorden, 2000) and the detection limits for the 2 methods are somewhat similar in foetal material (Kim et al., 2004; Mikami et al., 2005). The combination of stillbirths and mummified foetuses of different ages is mainly associated with intrauterine infections with viruses such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV) and encephalomyocarditis virus (EMCV) (Almond et al., 2006; Brewer et al., 2001). Furthermore, PPV and EMCV cause foetal lesions similar to PCV2. Thus, these infections are the main differential diagnosis to PCV2 associated reproductive failure.

Most reports on naturally occurring PCV2 associated reproductive failure have been descriptive case reports, focussing on clinical and pathological findings. These cases have been identified as incidental findings at the diagnostic laboratories and therefore lack detailed information on the time course of the outbreaks.

Here, we describe the first severe case of naturally occurring PCV2 associated reproductive failure in Denmark, where recordings of data over time in the affected herd, made it possible to study the progression of the foetal infection. Furthermore, in regard to PCV2 associated reproductive failure the diagnostic value of different techniques, including IHC for PCV2 detection, quantification of PCV2 DNA by real-time polymerase chain reaction (PCR) and total immunoglobulin G (IgG) measurements of foetal material, was revised. Finally, the pathogenesis of PCV2 was studied by examination of PCV2 replication, carried out by ISH of various foetal organs, including bone marrow.

Materials and methods

Case description

After experiencing severe problems with PMWS, a Danish specific pathogen free (SPF) farrow-to-wean production system stamped out the entire population and repopulated with 434 maiden and pregnant SPF gilts. The gilts were obtained from the same source, where they had been inseminated by semen from at least 56 different boars and were vaccinated against PPV. During a 2 month period, 133 gilts farrowed or aborted and the numbers of live born, stillborn and mummies were recorded (table 1 and figure 1). Six gilts aborted (5%, 6/133) between days 38 and 92 of gestation and 2 gilts died: 1 immediately after abortion and the other just before farrowing. Otherwise the affected gilts did not show any symptoms. The average reproductive performance parameters per litter, compared to Danish key figures (DKF) (Jultved, 2005), were: 10.9 live born (DKF 13), 2.5 stillborn (DKF 1.6) - 1.6 of the latter were mummies. All live born piglets seemed healthy and the preweaning mortality was 4.8% (DKF 14%).

The severe outbreak of reproductive failure started on the 23rd of March 2006 and the acute phase ceased around the 20th of April 2006 (figure 1), with the sows returning to normal reproductive performance during the following month. Major events occurring in the original and the new herd during the period from 27.11.2005 to 19.05.2006 are presented chronologically in figure 2.

Sampling

A total of 119 stillborn and mummified foetuses, including placenta samples, were collected and frozen at minus 20°C before submission to the National Veterinary Institute in Denmark for laboratory examination. Thirty-eight of these foetuses (cases) were suited for further examination

(table 2). Blood samples were taken from 26 maiden (n=11) or pregnant (n=9) gilts, and from gilts (n=6) which had aborted or delivered mummies. This group is from hereon referred to as "mature case pigs". Furthermore, 13 stillborn and mummified foetuses from another SPF farrow-to-wean production unit without a history of reproductive disorders, served as negative controls. From this herd 20 blood samples were taken from growing-finishing pigs (n=10), maiden (n=5) and pregnant (n=2) gilts, and sows (n=3). This group is from hereon referred to as "mature control pigs".

Necropsy and pathology

Placentas and foetal tissues were evaluated macroscopically. Foetal age in days (FA) was estimated for all foetuses (119 cases and 13 controls) according to Almond et al. (2006), i.e. foetal age in days = 21.07 + 3.11X, where X is crown to rump length (CR). The actual date where the foetuses had died, was calculated from the insemination date and FA for 106 of the case foetuses.

For histology, the following tissue samples were collected from the case foetuses: heart (n=38), liver (n=38), bone marrow (n=33), lungs (n=16) and kidneys (n=11). Additionally, tissue samples of heart, liver, lungs and kidneys, were taken from the control foetuses (n=13). All samples were fixed by immersion in 10 % neutral buffered formalin for 24 hours. After fixation, the bone marrow samples were decalcified 1 week in an aqueous solution of 14% ethylenediaminetetraacetic acid (EDTA) and 1.5% sodium hydroxide (NaOH), and subsequently washed in tap water. Next, all tissue samples were dehydrated, embedded in paraffin wax and cut in 3-5 μm thick sections. The sections were mounted on conventional glass slides and stained by hematoxylin and eosin, or mounted on SuperFrost®Plus slides (Mensel-gläser, Germany) for IHC and ISH.

The following tissue samples were collected from the foetuses and frozen at minus 40°C until further examination: heart (32 cases and 13 controls), liver (20 cases), lung (26 cases), kidney (3 cases), spleen (10 cases) and brain (8 cases). Additionally, samples of pleura cavity fluid (PCF) from the foetuses (38 cases and 13 controls) and serum samples from the mature pigs (26 cases and 20 controls), were frozen at minus 40°C until further examination.

Immunohistochemistry

Immunohistochemistry for PCV2 was carried out on all formalin fixed tissue samples from the case and control group. PCV2 was detected with a specific monoclonal antibody (McNeilly et al., 2001),

using a previously described method (Jensen et al., 2006). Positive reaction was scored as low, moderate or massive and the positive cells were morphologically characterized.

In situ hybridization

For detection of PCV2 replication, PCV2 IHC positive, formalin fixed samples of heart, liver and bone marrow from the case foetuses, were examined by ISH with a digoxigenin labelled oliogonucleotide probe. The probe was identical to the open reading frame 1 sequence of the PCV2 genome and detected the intermediary replicative form of PCV2, which is only present during viral replication (Pérez-Martín et al., 2007; Rosell et al., 1999). The ISH process with the replicative form probe (RFP) was slightly modified after Rosell et al. (1999). Positive reaction was scored as low, moderate or massive, and the positive cells were morphologically characterized.

Real-time PCR and nucleotide sequencing

DNA was extracted from the foetal heart tissue (32 cases and 13 controls) using QIAamp DNA Mini Kit (QIAGEN, Denmark) according to the manufacturer's instructions, and the level of PCV2 DNA was quantified with a Primer Probe Energy Transfer real-time PCR assay (Hjulsager et al., 2009). Results were expressed as number of PCV2 copies per 500 ng total DNA, where total DNA was measured spectrophotometrically (Nanodrop Technologies Inc., USA). The cut-off of the test was 10^4 PCV2 copies per 500 ng total DNA.

From 3 foetuses, originating from 3 different litters, the PCV2 genome was PCR amplified in 3 overlapping reactions and sequenced as previously described (Dupont et al., 2008).

Serology

To get an indication of intrauterine infection (Holler, 1994; Dalsgaard et al., 1979), the level of IgG was tested in PCF from all foetuses (38 cases and 13 controls) by enzyme linked immunosorbent assay (ELISA). The IgG levels were scored as: normal (below 25 μg/ml); indecisive (between 25-50 μg/ml); or elevated (over 50 μg/ml).

Serum from the mature pigs (26 cases and 20 controls) was tested for PCV2 antibodies by a non-commercial ELISA as described (Christensen et al., 2009).

Serum samples from the mature case pigs (n=26) were tested for presence of antibodies to PRRSV (EU and US type) by ELISA (Sorensen et al., 1997). The negative cut-off level was set at optical densities above 44 (OD% >44). The samples were also examined for antibodies to PPV by ELISA (Madsen et al., 1997) and *Leptospira* spp. by microscopic agglutination test (Friis et al., 2000).

Test for other agents

None of the animals were tested for *Brucella suis* or pseudorabies virus, as these infections at present are eliminated from Denmark. Material from selected case foetuses was cultured for bacteria and examined for presence of the following pathogens: EMCV, PPV, porcine enterovirus, swine influenza virus, adenovirus and *Leptospira* by diagnostic procedures, routinely performed at the laboratory of the National Veterinary Institute, Denmark.

On indication, i.e. presence of heart lesions and/or elevated IgG levels (Brewer et al., 2001), foetuses (n=10) from the affected herd were examined for EMCV by inoculation of cell cultures. The foetuses were divided into 3 groups and homogenates of pooled heart tissue and pools of spleen, were inoculated on porcine primary kidney and Vero cell cultures. Subsequently, after 1 passage the cell cultures were fixed and stained by antiserum for presence of EMCV and also PCV2, porcine enterovirus (types 1, 8 and 10), PPV, swine influenza virus and adenovirus type 3.

Pools of lung and liver from 20 foetuses were tested for PPV by antigen-ELISA (Jensen et al., 1998). Furthermore, foetal brain (n=8) or PCF (n=4) was tested for antibodies to PPV by immunofluorescence (IF) (Sorensen et al., 1980).

Investigation for *Leptospira* ssp. was carried out on foetal liver, kidney, lung, stomach or placenta by indirect IF (n=14) or cultivation (n=4), on indications like icterus and/or elevated IgG levels (Ellis, 2006). For IF a monoclonal rabbit-anti *L. bratislava* antibody was used for detection of *Leptospira interrogans* serovar *pomona* and *bratislava* (Friis et al., 2000).

Statistical analyses

Chi-square test, Fisher's exact test and/or Student's two sample t-test were used for analysing the data. All tests were carried out in SAS version 9.1 (SAS Institute, NC, USA). Statistical significant level was set at $P<0.05$.

Results

Pathology

The actual time of death could be estimated for 106 of all submitted foetuses, indicating that the earliest foetal deaths occurred in the end of January 2006/beginning of February 2006 and that 54% (57/106) of the foetuses were dead before the introduction to the new herd (figure 3). The condition and FA of the case foetuses not examined further was: 9 foetuses were in the initial stages of mummification (FA from 68-99, mean 93); 70 were totally mummified (FA from 30-93, mean 58); and no lesions were found in 2 foetuses (FA from 99-108, mean 105). Main pathologic findings of the case foetuses which were examined further are presented in table 2. Mean FA when these foetuses had died was 101 (±7). No signs of mummification or initial stages of mummification were observed in 7 and 31 of the foetuses, respectively. Other gross lesions included ascites (n=3), haemorrhage at the heart basis (n=3), dilated heart (n=2) and/or icterus (n=5). The foetal tissues were marked by the mummification process, showing moderate to severe tissue destruction. Histological lesions were detected in the myocardium of 39% (15/38) of the foetuses. The lesions were mainly focal or multifocal non-suppurative fibrotic myocarditis, sometimes accompanied by mineralization of the myofibrils (n=6) and/or non-suppurative epicarditis (n=4) (figure 4a). Non-suppurative perivasculitis was seen in the liver of 11 foetuses (figure 4b). No lesions were detected in bone marrow, lungs or kidneys. Suppurative placentitis was present in 1 placenta. Mean FA when the control foetuses had died was 105 (±10). Two of the control foetuses were in the initial stages of mummification, one had pale organs and another had a dilated heart. The remaining control foetuses and placentas were without macroscopic changes. No histological findings were observed in the control group. When comparing the case and control foetuses, there was no significant difference according to gross lesions, but histological lesions were more frequent among the case foetuses (P=0.002).

PCV2 IHC and RFP ISH

Among the case foetuses, IHC staining for PCV2 was positive in 37% (14/38), 24% (9/38) and 30% (10/33) of the examined hearts, livers and bone marrows, respectively (figure 4c-d). Furthermore, 6% (1/16) of the lungs and 18% (2/11) of the kidneys were positive for PCV2. The PCV2 loads in the positive organs were low (n=3), moderate (n=1) or massive (n=10) in the myocardium; low (n=6), moderate (n=1) or massive (n=2) in the liver; low (n=6), moderate (n=1) or massive (n=3) in the bone marrow; and low in the lung and kidney. Of the IHC positive animals where myocardium,

liver and bone marrow were available (n=13), the reaction was positive in all 3 organs of 54% (7/13) of the foetuses. The 14 IHC positive foetuses were delivered by 9 gilts; 8 of these gilts farrowed during a 9 days period (figure 2). The farrowing date of the last gilt was unknown. Mean FA where the foetuses had died, was 101 (± 6) and 101 (± 8) for the PCV2 IHC positive and negative case foetuses, respectively. This difference was not significant. For 11 of the IHC positive and 17 of the IHC negative foetuses, it was possible to identify the semen used for insemination, revealing that semen from 1 boar was significantly associated with 5 of the IHC positive foetuses (delivered in 3 litters) (P=0.005). All control foetuses were negative for PCV2 by IHC. No significant association was detected between gross lesions and positive IHC reaction, whereas histological lesions and positive IHC reaction was significantly associated (P<0.001).

All livers and bone marrows examined by the RFP ISH were negative, whereas 43% (6/14) of the examined hearts were labelled by the RFP, but only few cells in each section were positive, i.e. low grade reaction (figure 4e). The RFP positive hearts came from foetuses with low (n=1) and massive (n=5) PCV2 load (detected by IHC). The PCV2 IHC labelled the cytoplasm and sometimes the nucleus of the positive cells, whereas the RFP ISH only stained the nucleus (figure 4c-e). The cellular morphology of the labelled cells (IHC and ISH) were somewhat disrupted, because of the starting decay and dehydration of the tissues. However, in the myocardium it was possible to characterize most of the cells, positive by IHC and ISH, as myocardiocytes (figure 4c-e).

PCV2 PCR and serology

PCR results of the case foetuses are presented in table 2. When dividing the case foetuses into 2 groups according to PCV2 IHC results, the following PCR ranges were seen: 10^8 to 10^{12} for the IHC positive cases; and 0 to 10^9 for the IHC negative cases. The PCR results of the control group were below cut-off. When dividing the PCR values into categories so they enclosed most foetuses from 1 of the 3 groups, i.e. IHC positive, IHC negative or controls, the following categories came up: negative for PCV2 (DNA load below cut-off); suspected positive (DNA load 10^4-10^7); and positive for PCV2 (DNA load $>10^7$). All control foetuses and IHC positive foetuses were included in the negative and positive groups, respectively. The IHC negative cases (n=20) were included in the suspected positive group, except for 6 foetuses: 3 of these foetuses had PCR values below the cut-off and 3 had values above the category definition. When comparing the status of the foetuses,

i.e. IHC positive and negative cases, and controls, to the individual and categorized PCR results the associations were significant (P<0.001).

The total mean PCV2 antibody titers in the mature case pigs was 5812 (maiden gilts: 8432; pregnant gilts: 5894; gilts which had aborted or delivered mummies: 883). The mean PCV2 antibody titers of the age matched mature control pigs was 14010 (maiden gilts: 20250; pregnant gilts: 15650; sows: 3775), and in the remaining growing-finishing control pigs the mean was 47750. The antibody titers varied a lot between the animals in both the case and control groups. There was no significant difference between the total mean of the case group and the total mean of age matched mature control group.

Nucleotide sequencing of PCV2

The PCV2 sequences from the 3 case foetuses were compared in a phylogenetic tree to PCV2 sequences from a PMWS case-control study performed in Denmark in 2003/2004 (Dupont et al., 2008) and to sequences isolated from aborted porcine foetuses in Canada (Meehan et al., 2001) (figure 5). The results showed that PCV2 from the case foetuses were identical and belonged to genotype PCV-2b as all the PCV2 isolates from the Danish case-control study. No distinct clustering of PCV2 isolated from the case foetuses was found when compared to the case-control study. In contrast the aborted foetuses from Canada belonged to genotype PCV-2a.

Serology and other agents

In the case group, the total IgG level in the PCF was indecisive in 1 foetus and 10 foetuses had elevated levels (table 2). Two of the 14 PCV2 IHC positive foetuses had elevated IgG levels. No significant association was seen between PCV2 IHC results or PCR categories and IgG results of the case group. All foetuses in the control group had normal IgG levels in PCF. The frequency of foetuses with elevated IgG levels was higher in the case group compared to the control group (P=0.048).

All investigations of the foetal samples for EMCV, PPV, porcine enterovirus, swine influenza virus, adenovirus and *Leptospira* spp. were negative and no pathogenic bacteria were cultured. The sera from the mature case pigs had low antibody titers to PPV ranging from 50 to 512, except 1 pregnant

gilt, which had a titer of 2048. Likewise, the serum had no or low antibody titers to *L. bratislava* (titer range: 0-10) and was negative for antibodies to PRRSV (EU and US type).

Discussion

The results of the present study clearly show that the increase in number of mummified and stillborn foetuses, together with myocardial lesions and in situ detection of PCV2, correlates with the case definition of PCV2 associated reproductive failure (Segalés et al., 2006). Besides PCV2, we did not find indications of other abortion related infectious agents in the herd. The negative or low serum antibody titers to PPV, *L. bratislava* and PRRSV excluded recent infection with these agents.

All the IHC positive foetuses had PCR values above 10^8 PCV2 genomes per 500 ng DNA and most IHC negative foetuses had values below. This is in accordance with Brunborg et al. (2004), who reported that the IHC detection limit for PCV2 is 10^8 PCV2 genomes per 500 ng DNA. When the foetuses were divided into 3 groups and the PCV2 DNA loads were compared, the following significant associations were seen: IHC positive cases > IHC negative cases > control group. These results are in agreement with a similar study by Brunborg et al. (2007). The significant differences in PCV2 DNA load between the control and IHC negative groups indicate that some of the PCV2 IHC negative foetuses indeed were infected by PCV2. However, we can not ascertain if a low level of PCV2 cross contamination of the foetuses occurred in the field or during necropsy. Thus, we suggest that detection of more than 10^7 PCV2 genome copies per 500 ng DNA in tissues from aborted, stillborn and mummified foetuses is regarded as diagnostic for PCV2 associated reproductive failure and the detection of 10^4 to 10^7 PCV2 genome copies is indicative. Especially, when it comes to detection of PCV2 in severely dehydrated material from mummies, PCR offers an alternative to IHC, as routine tissue processing can be difficult of such material. Based on our and others results, we suggest that the finding of 1 foetus with more than 10^7 PCV2 genome copies per 500 ng DNA or detection of at least 2 foetuses with 10^4 to 10^7 PCV2 genome copies per 500 ng DNA, is sufficient for making the diagnosis of PCV2 associated reproductive failure.

Although the entire period of reproductive failure lasted for about 8 weeks, the PCV2 IHC positive foetuses were only delivered during a 9 days period early in the outbreak. This could result in diagnostic problems in practice, since reproductive failure often has lasted some time, before

samples are collected and send for laboratory examination. As a consequence, the role of PCV2 in reproductive failure might be underestimated, since previous studies of field material have reported a very low frequency of PCV2 positive samples from aborted foetuses (Bogdan et al., 2001; Maldonado et al., 2005). The clustering of IHC positive foetuses around a certain time point has not been reported from other field cases of PCV2 associated reproductive failure. Trying to elucidate the biological background for this clustering, at least 3 possible explanations arise. Firstly, the pregnant gilts could have become infected at the introduction to the new production facility. But as the first foetuses died in January/February 2006 and half of the foetuses were dead before the gilts were introduced into the new herd, this explanation is unlikely. Furthermore, the group of IHC positive foetuses would have been oldest at the time of infection compared to the IHC negative and thus, would be expected to be immunologically more mature. Another possibility is that the naïve gilts and foetuses became infected at insemination, as previous studies have shown that semen from boars contain PCV2 (Larochelle et al., 2000; McIntosh et al., 2006; Schmoll et al., 2008). Like this, Rose et al. (2007) showed that intrauterine inoculation with PCV2 at insemination could cause lesions indicative of PCV2 associated reproductive failure, resulting in delivery of mummies. In that study the CR of the mummies was 5-33 cm, showing that the foetuses had died over a protracted period from 37 to 124 days of gestation (DG), which was in agreement with the findings in our study. Furthermore, an association between a specific boar and foetal IHC reaction was detected, but this finding alone could not explain the clustering of all the IHC positive foetuses. The last hypothesis is that the gilts were horizontally infected in the original herd around the time of insemination and that the clinical outcome relied on the immunologic status of the gilts. As studies have shown that seroconversion to PCV2 occurs around 10-21 days post infection (Fort et al., 2007; Meerts et al., 2006), infection of naïve gilts just before or early in the gestation, would not give them time to respond to the infection. Thus, these gilts could transfer the full-blown PCV2 infection to their foetuses, which became IHC positive with high viral loads. Other simultaneously infected non-pregnant gilts would have some time to react to the infection, before they became pregnant. Thus, these gilts were able to reduce the viral load and partly protect their foetuses, which became IHC negative (and PCR positive), but still the infection was fatal to the foetuses. In summary, the fact that half of the foetal deaths occurred in the original herd makes the 2 last explanations the most plausible.

In the present study, 14% and 33% of the PCV2 IHC positive and negative case foetuses, respectively, had elevated levels of IgG. This difference was not significant, whereas the number of foetuses with elevated IgG levels in the case group were marginally higher compared to the control group. It was not expected to find normal IgG levels among most (86%) of the IHC positive foetuses. One explanation might be, that the foetuses were infected by PCV2 before they became immunocompetent (<70 DG) and therefore regarded the virus as "self", not producing antibodies against it. This has been shown experimentally by Pensaert et al. (2004), where foetuses inoculated with PCV2 at 57 DG did have viral DNA in the myocardium, but were negative for antibodies to PCV2. In general, foetuses infected after 70 DG should be able to mount a protective humoral immune response against infectious agents (Bachmann et al., 1975; Butler et al., 2001; Pensaert et al., 2004). Another explanation for the finding of foetuses that were IHC positive and had high levels of PCV2 DNA, but no antibodies, might be that they died quickly after the infection, before they seroconverted or they could have an impaired antibody response to PCV2 (Fort et al., 2007; Meerts et al., 2006). The finding of IHC negative case foetuses with PCV2 loads above 10^4 and normal or elevated levels of IgG could be expected in foetuses infected after 70 DG. These foetuses should be able to clear the virus and prevent further spread in the body by the production of neutralizing antibodies and the IgG level would depend on the antibody surplus. Such associations between PCV2 load and neutralizing antibody level, has been described in experimental studies of piglets (Fort et al., 2007; Meerts et al., 2005). Furthermore, Sanchez et al. (2001) found that foetuses inoculated with PCV2 after 70 DG had high levels of antibodies, no gross lesions and significantly less virus, compared to foetuses inoculated at 57 DG. In that study, it was suggested that the observed differences were due to the young foetuses being more susceptible to the infection or that the antibodies had interfered with the viral titers. Finally, though unlikely, the detected elevated IgG levels could result from intrauterine infection by pathogens other than PCV2. Altogether, IgG measurements are probably not very reliable as indication of intrauterine PCV2 infection.

Besides an increased number of abortions, mummies and stillborns, some field cases of PCV2 associated reproductive failure also reported an increase in number of weak born piglets, in which PCV2 could be detected by IHC (Brunborg et al., 2007; Josephson and Charbonneau, 2001; O'Connor et al., 2001). Like Pittman (2008) and West et al. (1999) we did not observe an increase in preweaning mortality during the outbreak, however, the PCV2 infection status of the live born

piglets was not examined in the present study. This difference in clinical outcome of PCV2 associated reproductive failure might depend on the immune responses of the individual foetuses, maternal immunocompetence, and/or the PCV2 genotype. In the present study, the PCV2 strains isolated from 3 foetuses from different sows belonged to genotype PCV-2b, as reported in another case of naturally occurring reproductive failure (Pittman, 2008). The viruses were identical and were closely related to the strains found in a PMWS case-control study in Denmark (Dupont et al., 2008), but different from previously described "abortion related strains" from Canada, which belonged to genotype PCV-2a (Farnham et al., 2003; Meehan et al., 2001). Furthermore, it has been possible to reproduce reproductive failure with a PCV-2a strain isolated from a case of PMWS (Yoon et al., 2004), thus, apparently no specific genotype is associated with reproductive failure.

The gross and histological lesions detected in the foetuses correspond with other reports (Brunborg et al., 2007; Mikami et al., 2005; Pittman, 2008). Furthermore, we detected a strong association between histological lesions and positive IHC staining. In agreement with our findings, most studies report that the myocardium seems to be the target organ in PCV2 associated reproductive failure (Brunborg et al., 2007; Pittman, 2008; Sanchez et al., 2003), though some did not find PCV2 in the heart, but instead in the lymphoid tissues and/or in the lungs (Kim et al., 2004; Park et al., 2005). Next after the heart, the bone marrow was the organ most frequently positive for PCV2, which was detected by IHC in 30% of the examined foetuses. The detection of PCV2 in the bone marrow of foetuses has not been described before. As the foetal tissues had started to dehydrate and decompose, most of the cells were destructed. Thus, it was not possible to identify the IHC positive cells by morphology, except in the heart, where the positive cells were recognized as myocardiocytes. Low grade viral replication was detected in 43% of the IHC positive hearts, in the nuclei of myocardiocytes. Replication was mainly seen in hearts with massive loads of PCV2 detected by IHC and PCR, the same association between massive loads of PCV2 and replication has been observed in thymuses from PMWS-affected pigs (Hansen et al., 2009). The finding of PCV2 in the bone marrow and viral replication in the hearts should be examined further in fresh tissues from experimental settings.

In conclusion, in this study of naturally occurring PCV2 associated reproductive failure, it was only possible to detect PCV2 by IHC in a short window of time early in the outbreak. Thus, IHC is only suited for diagnostics of acute stages of reproductive failure, whereas, quantitative PCR proved

valuable as a sensitive diagnostic method within a wider time span. Furthermore, it seems that IgG measurements in foetal PCF are not very reliable as indication of intrauterine infection with PCV2. Finally, in the foetuses, PCV2 was detected in the bone marrow and viral replication in the myocardiocytes. The impact of these findings should be elucidated further.

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References

Almond, G.W., Flowers, W.L., Batista, L., D'Allaire, S., 2006. Diseases of the Reproductive System. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine, Blackwell Publishing, Oxford, pp. 113-147.

Bachmann, P.A., Sheffy, B.E., Vauhan, J.T., 1975. Experimental in utero infection of fetal pigs with a porcine parvovirus. Infect. Immun. 12, 455-460.

Bogdan, J., West, K., Clark, E., Konoby, C., Haines, D., Allan, G., McNeilly, F., Meehan, B., Krakowka, S., Ellis, J.A., 2001. Association of porcine circovirus 2 with reproductive failure in pigs: a retrospective study, 1995-1998. Can. Vet. J. 42, 548-550.

Brewer, L.A., Lwamba, H.C., Murtaugh, M.P., Palmenberg, A.C., Brown, C., Njenga, M.K., 2001. Porcine encephalomyocarditis virus persists in pig myocardium and infects human myocardial cells. J. Virol. 75, 11621-11629.

Brunborg, I.M., Jonassen, C.M., Moldal, T., Bratberg, B., Lium, B., Koenen, F., Schonheit, 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. Invest. 19, 368-375.

Brunborg, I.M., Moldal, T., Jonassen, C.M., 2004. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. J. Virol. Methods 122, 171-178.

Butler, J.E., Sun, J., Weber, P., Ford, S.P., Rehakova, Z., Sinkora, J., Lager, K., 2001. Antibody repertoire development in fetal and neonatal piglets. IV. Switch recombination, primarily in fetal thymus, occurs independent of environmental antigen and is only weakly associated with repertoire diversification. J. Immunol. 167, 3239-3249.

Christensen, C.S., Bækbo, P., Bille-Hansen, V., Bøtner, A., Vigre, H., Enøe, C., Larsen, L.E., 2009. Induction of Porcine Postweaning Multisystemic Wasting Syndrome (PMWS) in pigs from PMWS unaffected herds following mingling with pigs from PMWS affected herds. Vet. Microbiol. Submitted.

Dalsgaard, K., Overby, E., Metzger, J.J., Basse, A., 1979. Rapid method for screening of immunoglobulins in porcine fetuses, using rocket immunoelectrophoresis. Application of an interspecies reaction between human and porcine mu-chain. Acta Vet. Scand. 20, 313-320.

Dupont, K., Nielsen, E.O., Baekbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Vet. Microbiol. 128, 56-64.

Ellis, W.A., 2006. Leptospirosis. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.). Diseases of Swine. Blackwell Publishing, Oxford, pp. 691-700.

Farnham, M.W., Choi, Y.K., Goyal, S.M., Joo, H.S., 2003. Isolation and characterization of porcine circovirus type-2 from sera of stillborn fetuses. Can. J. Vet. Res. 67, 108-113.

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, 244-255.

Friis, N.F., Jorsal, S.E., Sorensen, V., Schirmer, A.L., Lindahl, J., Thorup, F., 2000. Enzootics of Leptospira abortions in Danish sow herds practising loose housing on deep straw bedding. Acta Vet. Scand. 41, 387-390.

Hansen, M.S., Segalés, J., Fernandes, L.T., Grau-Roma, L., Bille-Hansen, V., Larsen, L.E., Nielsen, O.L., 2009. Detection of porcine circovirus type 2 and viral replication in primary lymphoid organs from naturally and experimentally infected pigs. Vet. Immunol. Immunopathol. Submitted.

Hjulsager, C.K., Grau-Roma, L., Sibila, M., Enoe, 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.

Holler, L.D., 1994. Diagnosis of swine abortion. J. Swine health prod. 2, 29-31.

Jensen, L., Jensen, T.K., Lind, P., Henriksen, S.A., Uggla, A., Bille-Hansen, V., 1998. Experimental porcine neosporosis. APMIS 106, 475-482.

Jensen, T.K., Vigre, H., Svensmark, B., Bille-Hansen, V., 2006. Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by Lawsonia intracellularis. J. Comp. Pathol. 135, 176-182.

Johnson, C.S., Joo, H.S., Direksin, K., Yoon, K.J., Choi, Y.K., 2002. Experimental in utero inoculation of late-term swine fetuses with porcine circovirus type 2. J. Vet. Diagn. Invest. 14, 507-512.

Josephson, G., Charbonneau, G., 2001. Case report of reproductive problem in a new startup operation. J. Swine Health Prod. 9, 258-259.

Jultved, C.R., 2005. Rapport over P-rapporternes resultater oktober 2005. Notat nr. 0532. Landsudvalget for Svin, Dansk Landbrugsrådgivning, Landscentret Svin. http://www.dansksvineproduktion.dk/Infosvin%20kilder/notater/2005/0532.html?id=3a0afad2-6a9d-4b26-b5a0-ae0ec3bc1403&ghostuid=36abdd01-05be-4290-b242-f70cd65d1439&templateuid=08004d2f-0f3b-47d9-a674-8785310e6190.

Kim, J., Jung, K., Chae, C., 2004. Prevalence of porcine circovirus type 2 in aborted fetuses and stillborn piglets. Vet. Rec. 155, 489-492.

Ladekjaer-Mikkelsen, A.S., Nielsen, J., Storgaard, T., Botner, A., Allan, G., McNeilly, F., 2001. Transplacental infection with PCV-2 associated with reproductive failure in a gilt. Vet. Rec. 148, 759-760.

Larochelle, R., Bielanski, A., Muller, P., Magar, R., 2000. PCR detection and evidence of shedding of porcine circovirus type 2 in boar semen. J. Clin. Microbiol. 38, 4629-4632.

Madsen, E.S., Madsen, K.G., Nielsen, J., Jensen, M.H., Lei, J.C., Have, P., 1997. Detection of antibodies against porcine parvovirus nonstructural protein NS1 may distinguish between vaccinated and infected pigs. Vet. Microbiol. 54, 1-16.

Maldonado, J., Segalés, J., Martinez-Puig, D., Calsamiglia, M., Riera, P., Domingo, M., Artigas, C., 2005. Identification of viral pathogens in aborted fetuses and stillborn piglets from cases of swine reproductive failure in Spain. Vet. J. 169, 454-456.

Mateusen, B., Maes, D.G., Van, S.A., Lefebvre, D., Nauwynck, H.J., 2007. Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. Theriogenology 68, 896-901.

Mateusen, B., Sanchez, R.E., Van, S.A., Meerts, P., Maes, D.G., Nauwynck, H.J., 2004. Susceptibility of pig embryos to porcine circovirus type 2 infection. Theriogenology 61, 91-101.

McIntosh, K.A., Harding, J.C., Parker, S., Ellis, J.A., Appleyard, G.D., 2006. Nested polymerase chain reaction detection and duration of porcine circovirus type 2 in semen with sperm morphological analysis from naturally infected boars. J. Vet. Diagn. Invest. 18, 380-384.

McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. Arch. Virol. 146, 909-922.

Meehan, B.M., McNeilly, F., McNair, I., Walker, I., Ellis, J.A., Krakowka, S., Allan, G.M., 2001. Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. Arch. Virol. 146, 835-842.

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Vet. Res. doi: 10.1186/1746-6148-2-6.

Meerts, P., Van Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. Viral Immunol. 18, 333-341.

Mikami, O., Nakajima, H., Kawashima, K., Yoshii, M., Nakajima, Y., 2005. Nonsuppurative myocarditis caused by porcine circovirus type 2 in a weak-born piglet. J. Vet. Med. Sci. 67, 735-738.

Nielsen, J., Ladekjær Hansen, A.S., Bille-Hansen, V., Lohse, L., Bøtner, A., 2004. PCV2-associated disease following intrauterine infection. Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany, p. 14.

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.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Invest. 19, 591-615.

Park, J.S., Kim, J., Ha, Y., Jung, K., Choi, C., Lim, J.K., Kim, S.H., Chae, C., 2005. Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. J. Comp. Pathol. 132, 139-144.

Pensaert, M.B., Sanchez, R.E., Jr., Ladekjaer-Mikkelsen, A.S., Allan, G.M., Nauwynck, H.J., 2004. Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. Vet. Microbiol. 98, 175-183.

Pérez-Martín, E., Rovira, A., Calsamiglia, M., Mankertz, A., Rodriguez, F., Segalés, J., 2007. A new method to identify cell types that support porcine circovirus type 2 replication in formalin-fixed, paraffin-embedded swine tissues. J. Virol. Methods 146, 86-95.

Pittman, J.S., 2008. Reproductive failure associated with porcine circovirus type 2 in gilts. J. Swine Health Prod. 16, 144-148.

Rose, N., Blanchard, P., Cariolet, R., Grasland, B., Amenna, N., Oger, A., Durand, B., Balasch, M., Jestin, A., Madec, F., 2007. Vaccination of porcine circovirus type 2 (PCV2)-infected sows against porcine Parvovirus (PPV) and Erysipelas: effect on post-weaning multisystemic wasting syndrome (PMWS) and on PCV2 genome load in the offspring. J. Comp. Pathol. 136, 133-144.

Rosell, C., Segalés, J., Plana-Duran, J., Balasch, M., Rodriguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J. Comp. Pathol. 120, 59-78.

Sanchez, R.E.Jr., 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.

Sanchez, R.E.Jr., 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, 169-176.

Schmoll, F., Lang, C., Steinrigl, A.S., Schulze, K., Kauffold, J., 2008. Prevalence of PCV2 in Austrian and German boars and semen used for artificial insemination. Theriogenology 69, 814-821.

Segalés, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. Anim. Health Res. Rev. 6, 119-142.

Segalés, J., Allan, G.M., Domingo, M., 2006. Porcine Circovirus Diseases. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine, Blackwell Publishing, Oxford, pp. 299-307.

Sorden, S.D., 2000. Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). Swine health prod. 8, 133-136.

Sorensen, K.J., Askaa, J., Dalsgaard, K., 1980. Assay for antibody in pig fetuses infected with porcine parvovirus. Acta Vet. Scand. 21, 312-317.

Sorensen, K.J., Botner, A., Madsen, E.S., Strandbygaard, B., Nielsen, J., 1997. Evaluation of a blocking Elisa for screening of antibodies against porcine reproductive and respiratory syndrome (PRRS) virus. Vet. Microbiol. 56, 1-8.

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. Diagn. Invest. 11, 530-532.

Yoon, K.J., Jepsen, R.J., Pogranichniy, R.M., Sorden, S., Stammer, R., Evans, L.E., 2004. A novel approach to intrauterine viral inoculation of swine using PCV type 2 as a model. Theriogenology 61, 1025-1037.

Tables

Table 1
Reproductive data of the gilts during the period from 23.03.2006 to 19.05.2006, divided into 4 sub-periods

Period	No. of live born	No. of mummies	No. of stillborn	% of mummies & stillborn*	% of failure deliveries†	Remarks
23.03-04.04	162	50	17	29% (67/229)	90% (18/20)	2 abortions 1 farrow: only mummies 2 farrows: all live born
05.04-13.04‡	117	59	8	36% (67/184)	93% (13/14)	1 farrow: only mummies 1 farrow: all live born
14.04-20.04	152	17	10	15% (27/179)	81% (13/16)	3 abortions 4 farrows: all live born
21.04-19.05	949	74	84	14% (158/1107)	63% (52/83)	1 abortion 1 farrow: only mummies 31 farrows: all live born
Total	1380	200	119	19% (319/1699)	72% (96/133)	

* Percentage of mummies and stillborn out of total number of born
† Percentage of abortions and deliveries with one or more mummified or stillborn foetus out of total deliveries
‡ 12/14 foetuses positive for porcine circovirus type 2 by immunohistochemistry were delivered during this period. The date of delivery for the remaining 2 foetuses was unknown

Table 2
Data of the case foetuses examined further
(Delivery date, foetus age in days, lesions, immunohistochemical (IHC) findings, levels of immunoglobulin G (IgG) and porcine circovirus type 2 (PCV2) DNA load tested by polymerase chain reaction (PCR))

				IHC for PCV2 in foetal tissues*						
Date†	Foetus age‡	Gross lesions§	Histological lesions¦	Heart	Liver	Bone marrow	Lung	Kidney	IgG#	PCV2 PCR¶
02.04	93	1	0	0	-	0	0	0	>100	1.2E+09
	93	1, 6	0	0	-	0	0	0	>100	7.9E+08
04.04	89	1	0	0	-	0	-	-	<25	BC
	96	1	0	0	-	0	-	-	<25	-
05.04	99	0	3	0	0	-	0	-	>50	-
	105	0	1, 2	++	+	-	0	-	<25	-
08.04	111	1	0	0	0	0	-	-	<25	1.1E+04
09.04	102	1, 5	1, 2	+++	+	+	-	-	<25	2.7E+12
	114	0	0	0	0	-	0	-	37	-
	108	0	0	0	0	-	0	-	>50	-
10.04	114	0	1	+++	0	-	0	-	>50	-
11.04	96	1, 3	2	+	0	+	-	-	<25	3.9E+12
12.04	96	1, 4, 5, 6	1, 2	+++	++	+	-	-	<25	2.2E+11
	96	0	1, 2	+++	+++	+++	-	-	<25	3.3E+12
13.04	102	1	1, 2	+++	+	0	0	0	<25	5.6E+10
	102	1	1, 2	+	0	+	-	-	<25	1.9E+12
	108	1, 4	1	+	0	0	0	+	>50	6.1E+08
	108	1	1, 2	+++	0	+	-	-	<25	8.3E+11
	96	1	1, 2	+++	+	++	-	-	<25	4.7E+09
	99	1, 3	1	+++	+	+++	-	-	<25	1.3E+12
	96	1, 6	0	0	0	0	-	-	<25	2.4E+06
	105	1	0	0	0	0	-	-	<25	3.8E+05
14.04	102	1	1	0	0	0	0	0	<25	BC
	108	0	0	0	0	0	0	0	<25	BC
	102	1	0	0	0	0	-	-	<25	1.3E+04
15.04	83	1	0	0	0	0	0	0	<25	7.5E+04
18.04	108	1, 3	0	0	0	0	0	0	>50	9.2E+07
	105	1, 6	0	0	0	0	0	0	>100	4.7E+04
05.05	105	1, 4	0	0	0	0	-	-	>100	2.3E+06
	105	1	0	0	0	0	-	-	<25	4.9E+05
06.05	102	1	0	0	0	0	-	-	<25	8.3E+04
	105	1	0	0	0	0	-	-	<25	2.1E+05
	99	1	0	0	0	0	0	0	>50	2.0E+06
	93	1, 6	1	0	0	0	-	-	<25	2.2E+05
	93	1	0	0	0	0	-	-	<25	2.5E+06
23.05	96	1	0	0	0	0	-	-	<25	1.8E+06
**	96	1	1, 2	+++	+	+	-	-	<25	9.9E+11
**	96	1	1, 2	+++	+++	+++	+	+	<25	4.7E+12

* The IHC reaction was scored as absent (0), low (+), moderate (++) or massive (+++). Not examined (-)
† Date = date of delivery
‡ Foetus age: foetal age in days calculated on basis of measured crown to rump length (data not shown). See text for details
§ Gross lesions: 0: no gross lesions; 1: initial stage of mummification; 2: totally mummified; 3: ascites; 4: haemorrhage at the basis of the heart; 5: dilatation of the heart; 6: icterus
¦ Histological lesions: 0: no histological lesions; 1: non-suppurative myocarditis; 2: non-suppurative perivasculitis in the liver; 3: suppurative placentitis
The IgG levels were scored as normal (<25 μg/ml), indecisive (25-50 μg/ml) or elevated (>50 μg/ml)
¶ PCV2 copies per 500 ng total DNA. BC = below cut-off. Not tested (-)
** These foetuses were delivered by the same gilt, the delivery date was unknown

Figure legends

Fig. 1. Percentage of stillborn, live born and mummies delivered by 127 gilts during the period from 23.03.2006 to 19.05.2006.

Fig. 2. Major events in the original and new herd during the period from 27.11.2005 to 19.05.2006 presented chronologically.

Fig. 3. Estimated date of death for 106 of the stillborn and mummified foetuses that were delivered during the period from 23.3.2006 to 19.05.2006.

Fig. 4. Histology of tissues from mummified and stillborn foetuses.

(a) Fibrotic non-suppurative myocarditis with mineralization (m). Infiltrating mononuclear cells (l). Hematoxylin and eosin (HE), scale bar = 62 μm.

(b) Non-suppurative perivasculitis in the liver. Infiltrating mononuclear cells (l). Liver parenchyma (p). HE, scale bar = 32 μm.

(c) Immunohistochemistry (IHC) for porcine circovirus type 2 (PCV2) in the heart. Positive reaction (red) in nucleus (arrow) and cytoplasm of myocardiocytes (insert). Scale bar = 32 μm. Insert, scale bar = 10 μm. Chromogen AEC and hematoxylin counterstain.

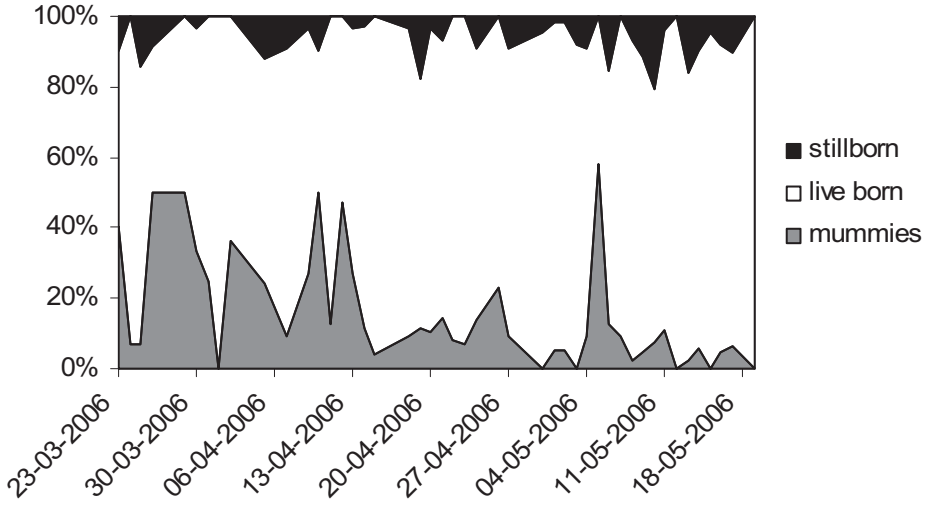
(d) Positive IHC reaction for PCV2 in the cytoplasm of cells (arrow) in the bone marrow. Chromogen AEC and hematoxylin counterstain, scale bar = 17 μm.

(e) In situ hybridization (ISH) for replication of PCV2 in the heart. Positive reaction (dark blue) in nucleus of myocardiocytes. Tissues are more or less (insert) affected by the mummification and ISH process. Scale bar = 10 μm, chromogen NBT/BCIP and fast green counterstain.

Fig. 5. Phylogenic analysis of full-length PCV2 genomes from the Danish (DK) case-control study (Dupont et al., 2008), the Danish mummified foetuses (Abort-2006-DK) from this study and the Canadian (CAN) sow abortions (Meehan et al., 2001). PCV2 from the Danish case-control study and the Danish mummified foetuses belonged to genotype PCV-2b, whereas PCV2 from the Canadian abortions belonged to genotype PCV-2a. Genbank accession numbers are given for the PCV2 sequences. The rooted phylogenetic tree was constructed by the neighbour-joining method. Significant bootstrap values are indicated as a percentage for 1000 replicates.

Figures

Figure 1



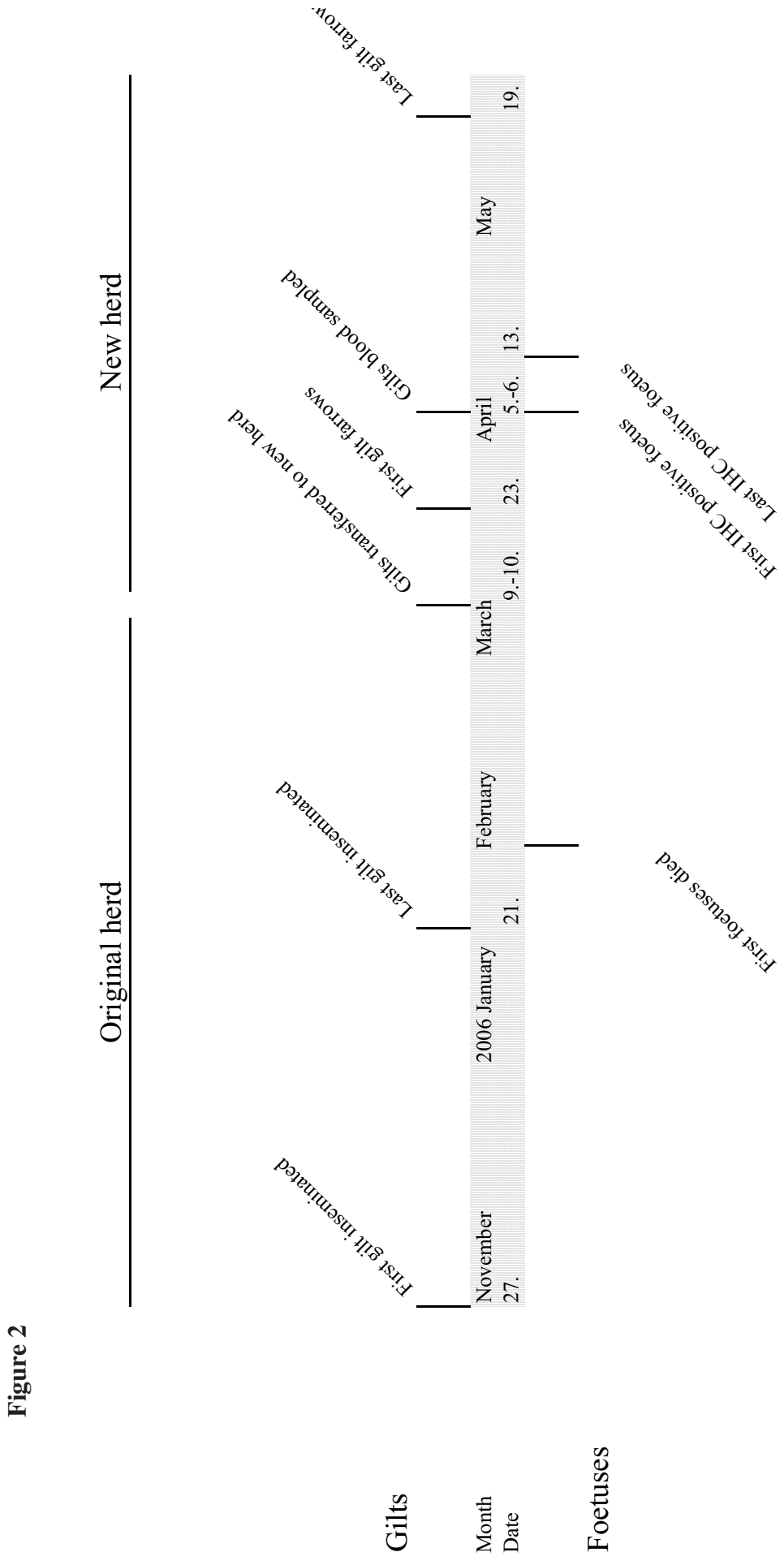

Original herd
New herd
Gilts
Month
Date
Foetuses
November
27.
2006 January
21.
February
March
9.-10.
23.
April
5.-6.
13.
May
19.
First gilt inseminated
Last gilt inseminated
Gilts transferred to new herd
First gilt farrows
Gilts blood sampled
Last gilt farrow
First foetuses died
First IHC positive foetus
Last IHC positive foetus

Figure 2

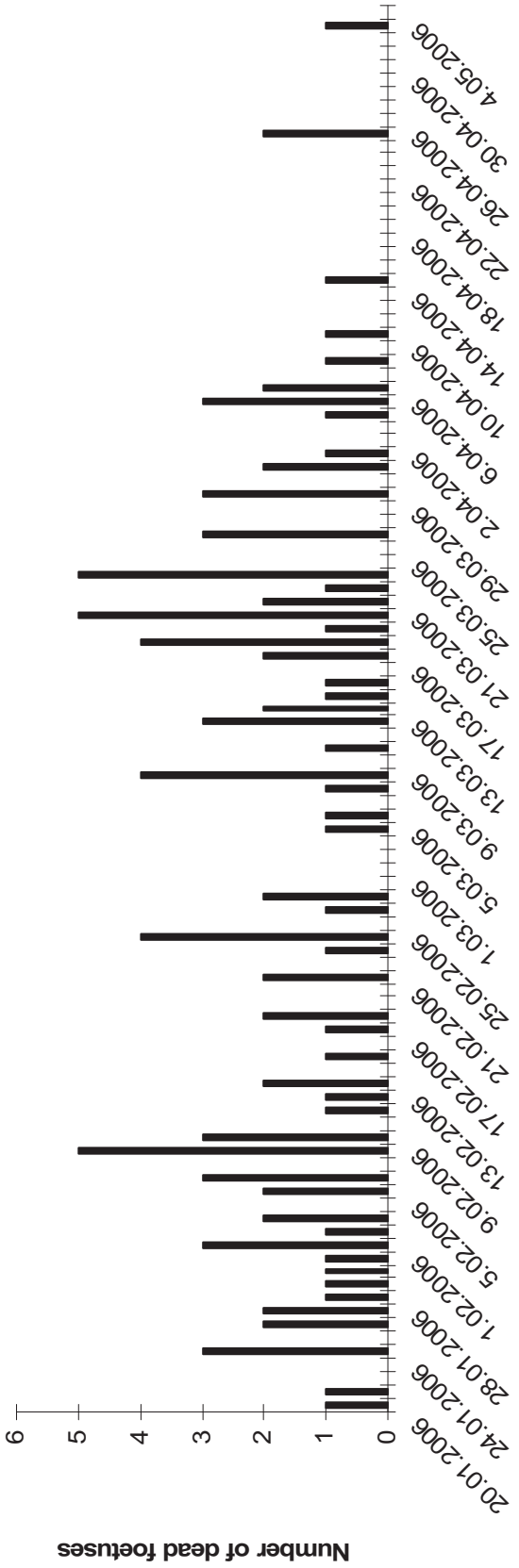

Number of dead foetuses
0
1
2
3
4
5
6
20.01.2006
24.01.2006
28.01.2006
1.02.2006
5.02.2006
9.02.2006
13.02.2006
17.02.2006
21.02.2006
25.02.2006
1.03.2006
5.03.2006
9.03.2006
13.03.2006
17.03.2006
21.03.2006
25.03.2006
29.03.2006
2.04.2006
6.04.2006
10.04.2006
14.04.2006
18.04.2006
22.04.2006
26.04.2006
30.04.2006
4.05.2006

Figure 3

Figure 4

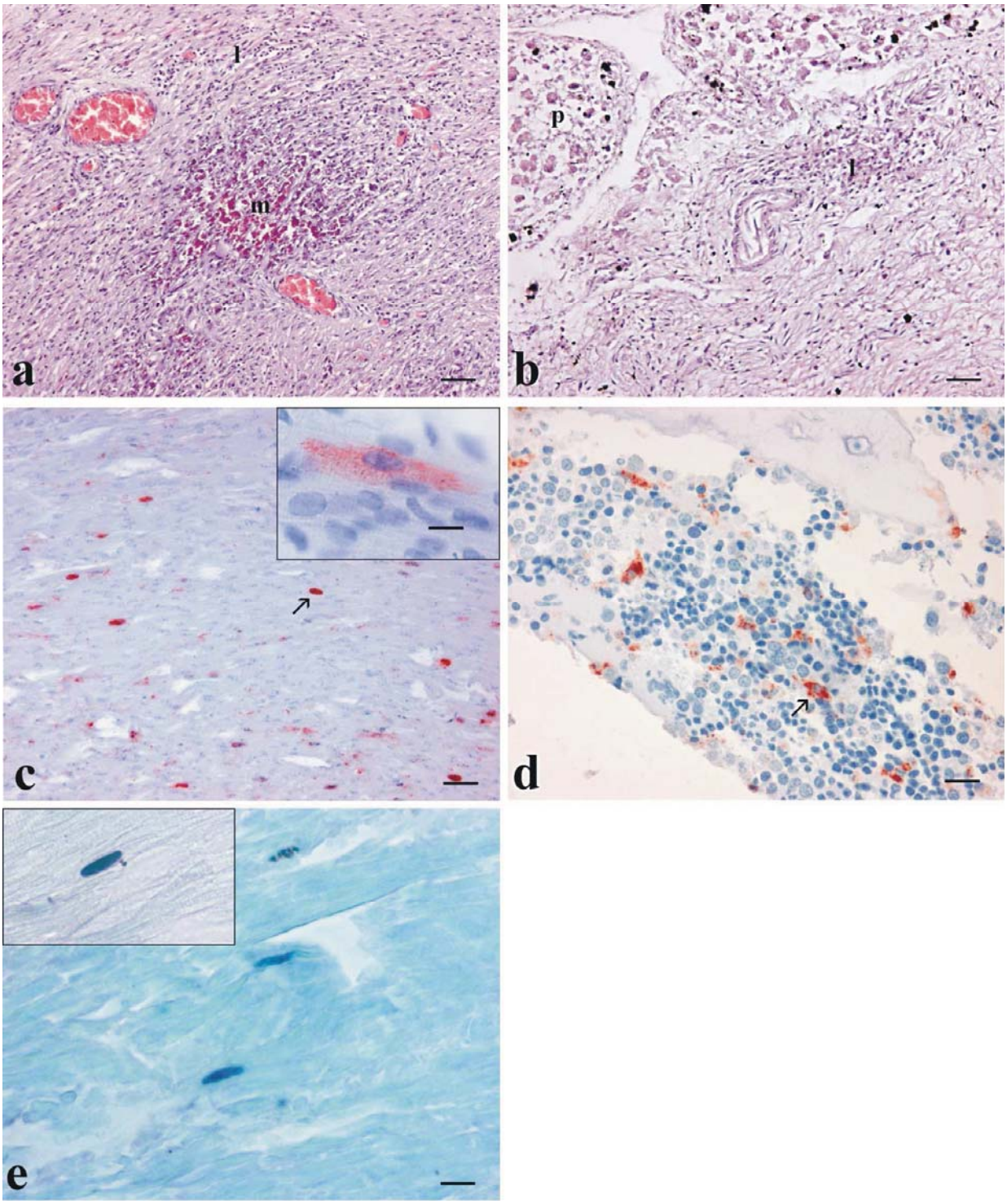
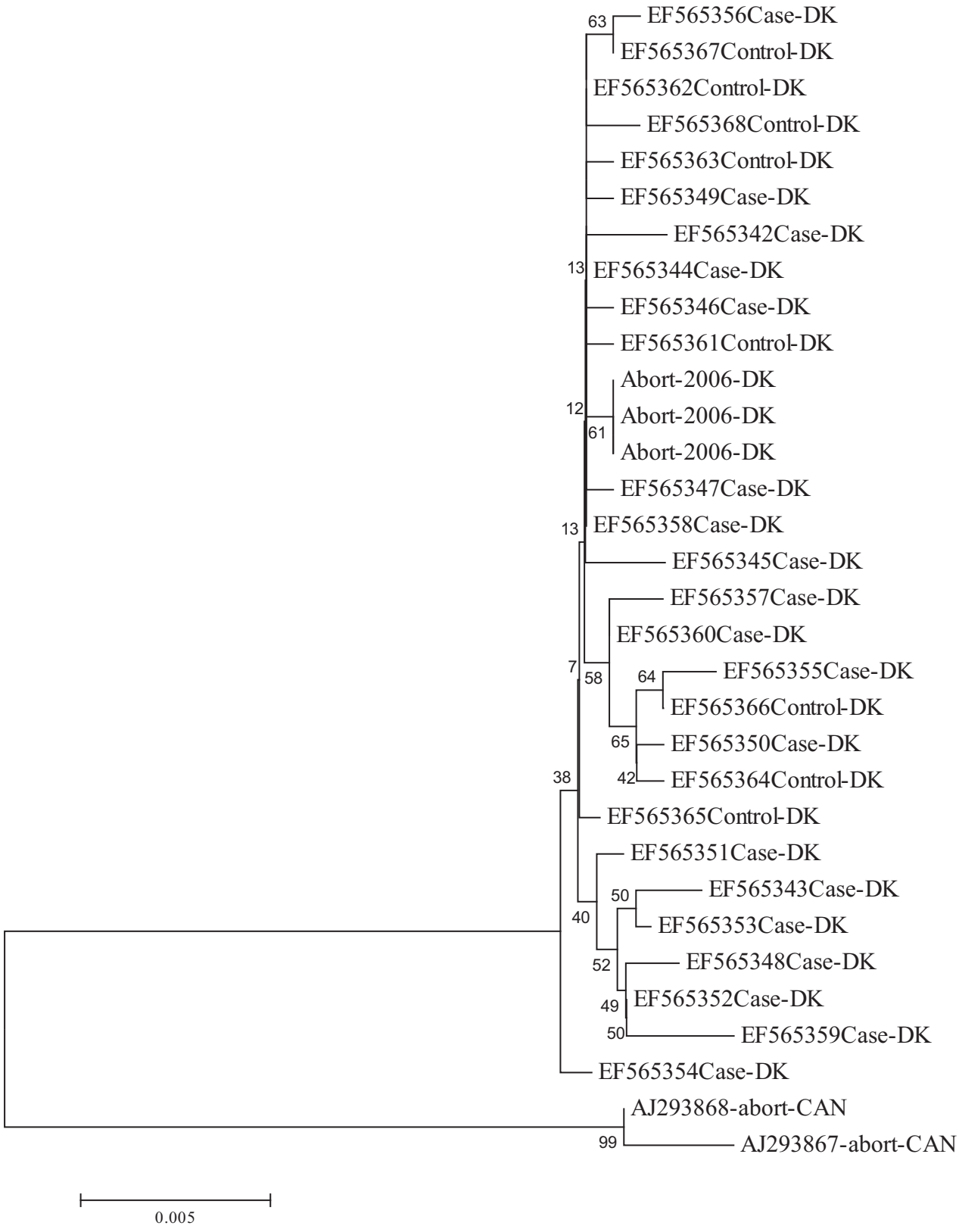


Figure 5


63
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EF565346Case-DK
EF565361Control-DK
Abort-2006-DK
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Abort-2006-DK
61
Abort-2006-DK
EF565347Case-DK
13
EF565358Case-DK
EF565345Case-DK
EF565357Case-DK
EF565360Case-DK
7
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EF565366Control-DK
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EF565351Case-DK
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EF565343Case-DK
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49
EF565352Case-DK
50
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EF565354Case-DK
AJ293868-abort-CAN
99
AJ293867-abort-CAN
0.005

Manuscript IV

Porcine respiratory disease complex in Danish finishing pigs - update on pathology and pathogens

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Porcine respiratory disease complex in Danish finishing pigs - update on pathology and pathogens

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Summary

Respiratory infections are some of the most important disease entities in growing pigs. In order to elucidate the multifactorial aetiology of porcine respiratory disease complex (PRDC) in Denmark, lungs from 148 finishing pigs with cranioventral bronchopneumonia (case group) and 60 pigs without lung lesions (control group) were collected at Danish abattoirs. The pathogens involved in PRDC, and their interactions were identified and linked to the histopathological diagnosis in order to broaden the understanding of the pathogenesis of porcine pneumonia. The lung samples were cultivated for bacteria and tested by multiplex polymerase chain reaction for presence of swine influenza virus (type A), porcine reproduction and respiratory syndrome virus (both EU and US type), porcine circovirus type 2 (PCV2), porcine respiratory coronavirus, porcine cytomegalovirus, *Mycoplasma hyopneumoniae,* and *Mycoplasma hyorhinis*. All gross lesions in the case group were cranioventral, lobular bronchopneumonia, consistent with PRDC. The histopathological examinations revealed acute (n=10), subacute (n=24) and chronic (n=114) cases of bronchopneumonia, and a broad variety of lesions was found, often in coexistence. A total of 5 bacterial species, 5 viruses and 2 mycoplasma species was detected, in different combinations. PCV2, *M. hyopneumoniae*, *M. hyorhinis* and *Pasteurella multocida* were detected most frequently among the PRDC affected swine, and the diversity and number of pathogens were higher in these animals compared to the control group. In some specific lesions particular pathogens occurred more frequently, however no clear-cut associations were detected between pathogens and histological

lesions or histopathological diagnoses. In conclusion, PRDC occurs more frequently than enzootic pneumonia among Danish finishing pigs. The histopathology of PRDC is complex and nearly all pulmonary reaction patterns can be observed.

Keywords: porcine respiratory disease complex (PRDC); bronchopneumonia; histopathology; swine

Introduction

Respiratory infections constitute some of the most important diseases in growing pigs, resulting in substantial economic losses and reduced welfare (Sørensen *et al.*, 2006; Thacker, 2001). Porcine respiratory disease complex (PRDC) is a multifactorial disease entity affecting finishing pigs from 14 to 22 weeks of age (Kim *et al.*, 2003; Thacker, 2001). Morbidity ranges from 30-70% and mortality varies from 2-20% (Halbur, 1998; Harding and Halbur, 2002; Harms *et al.*, 2002). Lesions are primarily located in the cranioventral parts of the lung, where consolidation, discoloration and failure of the lung tissue to collapse might be observed (Harms *et al.*, 2002). Histopathology may vary according to the involved pathogens, but bronchopneumonia, sometimes in combination with interstitial pneumonia, is often reported (Harms *et al.*, 2002; Kim *et al.*, 2003). In the US, the most commonly isolated pathogens are porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine circovirus type 2 (PCV2), *Pasteurella multocida* and *Mycoplasma hyopneumoniae*. Other important pathogens associated with PRDC are *Streptococcus suis*, *Actinobacillus pleuropneumoniae* and [*Haemophilus*] *parasuis* (Choi *et al.*, 2003; Harms *et al.*, 2002; Thacker, 2001). A study from 1999 showed that 25% of the Danish finishing pigs had cranioventral bronchopneumonia (CBP) at slaughter (Christensen and Enoe, 1999). Contemporary abattoir surveys in Switzerland and Belgium showed a similar prevalence (Grest *et al.*, 1997; Maes *et al.*, 2001), whereas earlier surveys revealed a higher prevalence, from 37% in Canada (Osborne *et al.*, 1981), 45% in Australia (Davies *et al.*, 1992) to 78% in another Canadian study (Wilson *et al.*, 1986). Abattoir surveys of porcine bronchopneumonia usually focus on the gross pathology and/or the microbiology, whereas the histopathology and microbiology is seldom linked. A survey of the pathogens involved in CBP in Danish pigs has not been performed for decades, and since new respiratory viruses, i.e. PCV2, porcine respiratory coronavirus (PRCV), PRRSV and new strains of SIV, have been introduced in recent years, a survey of the respiratory pathogens has become relevant.

The objectives of the present study were to elucidate the complexity of the pathogens involved in PRDC and their interactions in finishing pigs; and to associate these findings with the histopathological diagnosis, in order to broaden the understanding of the pathogenesis of porcine pneumonia.

Materials and Methods

Animals

During spring 2006 and winter 2007, lungs including tracheo-bronchial lymph nodes, from 148 finishing pigs with CBP (cases) and 60 pigs without macroscopic lung lesions (controls) were collected from two Danish abattoirs in Zealand and Jutland. Cases of CBP were defined as those with lesions affecting a minimum of three cranioventral lung lobes, i.e. apical, cardiac and intermediate lobes (Sørensen *et al.*, 2006). The lesions were acute and chronic inflammation and included consolidation, swelling, hyperaemia, failure to collapse and/or fibrosis. The collected material was cooled and processed (see below) within 5 hours after sampling. Tracheo-bronchial lymph nodes were not obtained from 2 animals with CBP and 5 control animals.

Gross Pathology

Lungs and tracheo-bronchial lymph nodes were evaluated macroscopically, including the morphological pattern, type of exudate and age of the pulmonary lesions, and a preliminary diagnosis was recorded. Lymph nodes were scored according to size: normal (0), moderate enlargement (1) or marked enlargement (2).

Histopathology

Tissue samples were taken from lung lesions (cases), normal appearing lung tissue from the dorsal surface of the right diaphragmatic lobe (all controls) and from the right apical lobe (20 controls). The samples were fixed by immersion in 10% neutral buffered formalin for 24 hours. The samples were subsequently dehydrated, embedded in paraffin wax, cut in 3-5 µm thick sections, mounted on conventional glass slides and stained with haematoxylin & eosin (Stevens and Wilson, 1996). Tissue sections for immunohistochemistry (IHC) were mounted on SuperFrost®Plus slides (Mensel-gläser, Braunschweig, Germany). Selected sections were stained by Masson trichrome and phosphotungstic acid hematoxylin (Luna, 1968) to detect fibrin and collageneous tissue. IHC for

cytokeratin (Soerensen *et al.*, 2005) was applied to confirm the presence of epithelial hyperplasia, hyperplasia of type II pneumocytes and atelectasis.

Sections were examined systematically by evaluation of the following structures in each section: bronchi, bronchioles and bronchus-associated lymphoid tissue (BALT); alveolar ducts and alveoli, including alveolar septa; peribronchial, peribronchiolar and interlobular connective tissues; and pleura. BALT hyperplasia was graded as follows (Ross, 1999): absent (0); mild (+) i.e. diffuse infiltration of lymphocytes in the peribronchial, peribronchiolar and perivascular tissues including the lamina propria of the airways; moderate (++) i.e. increased diffuse infiltration of lymphocytes and/or presence of a few lymphoid nodules; marked (+++) i.e. considerable number of lymphoid nodules; or extensive (++++) i.e. extensive number of lymphoid nodules affecting most of the lung section (figures 1a-d). Alveolar exudates were classified according to Bochsler and Slauson (2002) as suppurative, i.e. neutrophils dominated; non-suppurative, i.e. mononuclear inflammatory cells dominated; or mixed, i.e. intermediate grades between suppurative and non-suppurative (figures 1e-g). Hyperplasia of type II pneumocytes was present when they lined more than 3% of the alveolar surface area (Plopper and Adams, 2006) (figure 1h). Crowding of type II pneumocytes in close proximity to the interlobular connective tissue was not considered a significant lesion. Acute lesions were those with neutrophils as the dominating inflammatory cell type, extensive oedema and/or fibrin exudation, and absence of chronic signs. Chronic signs were fibroplasia; BALT hyperplasia grade ++ to ++++; hyperplasia of bronchial or bronchiolar epithelium; presence of bronchiolar polyps and alveolar polyp-like structures with a fibrous core; hypertrophy of the smooth muscle layer around bronchioles and alveolar ducts; and cellular infiltrates primarily consisting of lymphocytes and plasma cells. Lesions, which were not entirely acute or chronic, e.g. cellular infiltration dominated by neutrophils, combined with moderate BALT hyperplasia as the only chronic lesion, were regarded as subacute. Thus, the extent of acute vs. chronic signs determined the age of the inflammation. The histopathological diagnosis was regarded as the definitive diagnosis.

Microbiology

Bacterial culture

Swabs from lung lesions (cases) and from macroscopically normal lung tissue of the right diaphragmatic lobe (controls) were sampled under sterile conditions, plated on blood agar plates

(Blood agar Base CM 55, Oxoid, Basingstoke, United Kingdom with 5 % calf blood) and incubated aerobically in a sealed plastic bag at 37°C for 18-24 hours. All bacteriology samples were cross-inoculated with a v-factor producing *Acinetobacter calcoaeceticus*. Bacterial isolates were identified using standard methods for phenotypic characterization as previously described (Barrow and Feltham, 1993). Growth of *P. multocida* was evaluated semi-quantitatively according to the number of bacterial colonies on the primary plates: weak growth (+) (1-25 colonies), dense growth (++) (25-300 colonies) or massive growth (+++) (>300 colonies). *Streptococcus* spp. were identified by Pulsed Field Gel Electrophoresis and strains representing different genotypes were 16S rRNA sequenced (Chadfield *et al.*, 2004). All v-factor dependent cultures were also subjected to 16S rRNA sequencing to obtain a final identification. Mixed cultures demonstrating less than 25 colonies were regarded as contamination.

Mycoplasma and virus detection by polymerase chain reaction (PCR)

Tissue samples from lung lesions (cases) and macroscopically normal lung tissue (controls) were frozen at -20°C and investigated by multiplex PCR (Pulmotest®, Landeslabor Schleswig-Holstein, Food-, Veterinary, and Environmental Diagnostic Institute of Schleswig-Holstein, Neumünster, Deutschland) according to the method described by Harder and Huebert (2004). Pathogens detected by the multiplex PCR were: SIV (influenza A), PRRSV (both EU and US type), PCV2, PRCV, porcine cytomegalovirus (PCMV), *M. hyopneumoniae,* and *M. hyorhinis*.

Statistics

Data were primarily analyzed statistically by Chi-square test or by Fisher's exact test when a small sample size necessitated this. The analysis of number of pathogens in relation to lesion age groups was carried out as a Poisson regression. The degree of association was estimated by Spearman rho factor. All analyses were done in SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) with the significance level set at $P<0.05$. Statistical analysis was not carried out on groups that included less than 9 pigs, and for this reason the results for PRRSV EU and US types were pooled. When looking for associations between pathogens or combination of pathogens and lesion age group, type of exudate, or histopathological diagnosis group, we tested against the remaining cases of CBP, excluding the control animals.

Results

Gross pathology

The gross lesions of all lungs in the case group (n=148) were consistent with cranioventral, lobular bronchopneumonia (figure 2a). Mucopurulent, purulent or seromucous exudates were found in all cases of CBP, which were distributed into the following lesion ages: 7% (11/148) acute, 18% (26/148) subacute and 75% (111/148) chronic cases. Focal or multifocal fibrotic pleuritis of the diaphragm lobes, without any relation to the pneumonic lesions, was present in 36% (54/148) of these animals. Except for 13% (8/60) of the control pigs, which had chronic focal pleuritis, none of the controls had gross lesions. No macroscopic lesions were detected in the lymph nodes in any of the groups. Lymph node scores for the cases of CBP were: score 0, 3% (4/146); score 1, 66% (96/146); and score 2, 32% (46/146) and for the control pigs: score 0, 62% (34/55); score 1, 35% (19/55); and score 2, 4% (2/55). A significant association was present between lymph node enlargement and CBP ($P<0.001$). When the lymph node scores were compared to the lesion age group (data not shown), no significant association was found ($P=0.16$).

Histopathology

The histopathological diagnoses of the case group are presented in table 1, and the main histopathological findings are presented in table 2. Histological lesions were found in the alveoli of all lungs with CBP. The majority also had lesions in bronchioles and/or alveolar ducts, often together with lesions in the interlobular connective tissue. Bronchitis and bronchiolitis were mainly suppurative with concurrent epithelium hyperplasia in the subacute and chronic cases (figure 2b). In cases of bronchiolitis, destruction of the epithelial lining in the bronchioles was observed in 17% (1/6), 50% (4/8) and 37% (11/30) of the acute, subacute, and chronic cases of CBP, respectively. The main finding in the alveoli was infiltration of inflammatory cells (neutrophils, lymphocytes, plasma cells) reflecting the age of inflammation, whereas macrophages were identified in all stages. Oedematous flooding was a common finding in alveoli, in acute, subacute, and chronic cases of CBP, with the degree of oedema being more pronounced in acute stages (figure 2c). Varying degrees of thickening of the alveolar septa, mainly by collagenous tissue, were seen in 25% (37/148) cases of CBP (figure 2d). Alveolar polyp-like structures could be seen in acute, subacute and chronic bronchopneumonia, whereas bronchiolar polyps only occurred in subacute and chronic lesions. Alveolar polyp-like structures, with a fibrinous core could be observed in both acute and chronic cases, whereas polyps consisting of collagen were not present in acute cases (figures 2e-f).

The major findings in the interlobular connective tissues were oedema, diffuse lymphocyte infiltration and/or fibroplasia. Other findings were hypertrophy of the muscles around bronchioles and alveolar ducts, and hyperplasia of type II pneumocytes. Occasional findings were vasculitis, thrombosis and areas of necrosis tissue with or without mineralization (figure 2g). Not all combinations of the above described lesions were seen in the lung sections. The lungs from 3 animals, diagnosed as acute (n=1) or subacute (n=2) according to gross pathology, were definitely diagnosed as chronic, on basis of histopathology. Histological lung lesions were seen in 50% (30/60) of the control animals, and no difference was observed between the samples from the diaphragmatic and the cranioventral lobes. The findings mainly included: focal or multifocal thickening of alveolar septa (figure 2h) and/or mild BALT hyperplasia (table 2).

Microbiology

A total of 12 potential pathogens (5 bacterial species, 5 viruses, 2 mycoplasma species) was detected and these are shown in table 3. In general, the frequencies of the pathogens were higher in the case group, compared to the control group, except for PCMV, which was more frequent in the control group (though not significant). PRCV was not detected in any of the pigs. *Staphylococcus aureus*, [*H.*] *parasuis*, *A. pleuropneumoniae* and SIV-A were only detected in 1 to 5 animals. *S. aureus*, [*H.*] *parasuis*, *A. pleuropneumoniae*, PRRSV EU and US type were only found in the cases of CBP. When comparing cases and controls according to pathogens (table 3), all grades of *P. multocida* ($P<0.001$ to $P=0.004$), *M. hyopneumoniae* ($P<0.001$) and *M. hyorhinis* ($P<0.001$) were found more frequently in the cases of CBP. Furthermore, the finding of mixed cultures and sterile samples was more frequent in the control group than in the case group ($P<0.001$). Significant associations were observed between 8 pairs of pathogens among the diseased animals and between 2 pathogens in the control group, the results are presented in table 4.

A total of 63 different combinations of pathogens was present, many of which only included one animal (n=34) (data not shown). Among the cases of CBP and the control animals there were 51 and 23 different combinations of pathogens, respectively. The 10 most frequent combinations are presented in table 5. Some combinations were seen in either diseased or healthy animals. The 3 most frequent combinations, only isolated from cases of CBP, were: combination no. 1, 6 and 10 (table 5). Likewise, the two most frequent combinations, only seen among the control animals, were: mixed culture and PCV2; and PCV2 as solitary pathogen.

Associations between microbiological and histopathological findings

When testing association of selected histological lesions (bronchiolar polyps, BALT hyperplasia, fibrin exudation into alveoli, hyperplasia of type II pneumocytes, thickening of alveolar septa, alveolar polyp-like structures, necrotic foci) within the case group, and subsequently within lesion age groups, to pathogens, significant results were obtained for the following: in chronic cases with bronchiolar polyps, *P. multocida* (+++) was isolated more frequently (P=0.016); BALT hyperplasia (grade ++ to ++++) was associated with PCMV in chronic cases (P<0.001); fibrin exudation into alveoli was associated with the isolation of *P. multocida* (++) (P=0.018); thickening of alveolar septa was never seen when *P. multocida* (+) was isolated (P=0.013). Furthermore, in 78% (7/9) of cases with necrotic foci, *P. multocida* (+++) was present, although only borderline associated (P=0.062). No association between histological lesions and pathogens was found in the control group.

Table 6 presents the frequencies of single pathogens, divided according to the diagnosis groups. *P. multocida* was, as the only pathogen, isolated more frequently from the following groups: *P. multocida* (++) and subacute suppurative bronchopneumonia (SSBP) (P=0.054); *P. multocida* (++) and subacute mixed bronchopneumonia (SMBP) (P=0.037); and *P. multocida* (+) and chronic non-suppurative bronchopneumonia (CNBP) (P=0.043). *S. suis* was not isolated from any cases of acute suppurative bronchopneumonia (ASBP) (P=0.021). Significant associations were not seen, when comparing single pathogens and type of exudate (suppurative, mixed and non-suppurative), or lesion age groups (acute, subacute and chronic).

In figure 3 the count of pathogens detected in each pig is listed for the lesion age groups and the control group. At least one pathogen was detected in all lung samples (cases and controls). Detection of a single pathogen (PCV2) occurred in 7 animals, all from the control group. The majority of pigs in the control group had a significantly lower count of pathogens, while cases of CBP had increased counts (P<0.001). Between lesion age groups there was a tendency to a lower count in the chronic cases compared to acute and subacute cases. However, this difference was not significant.

When comparing the 10 most frequent combinations of pathogens and lesion age groups (table 5) the only significant association was combination no. 3, which was seen more frequently in the acute

cases (30%, 3/10) (P=0.044), than in the subacute (13%, 3/24) and chronic cases (7%, 8/114). There were no significant associations, when comparing these combinations of pathogens with types of exudate or histopathological diagnosis (data not shown).

Discussion

Pneumonia can be classified as embolic, bronchopneumonia and interstitial or bronchointerstitial (Caswell and Williams, 2007), based on the morphologic pattern observed in experimental studies and/or infections with single pathogens. In the present study of naturally occurring pneumonia, we saw aspects of different pneumonic lesions, mainly bronchopneumonia associated with thickening of the alveolar septa. Furthermore, we observed chronic lesions accompanied by acute lesions, which may represent a chronic active nature of the respiratory disease or two different disease incidents. With a necessity to separate bronchopneumonia according to the age of disease, i.e. acute, subacute or chronic, we evaluated the histological basis for each of these stages, bearing in mind the possibility of misinterpretation. Only few studies have focused on the histopathological findings in porcine lungs with naturally occurring PRDC (Harms *et al.*, 2002), whereas many studies either describe naturally occurring gross lesions or histopathology of experimental co-infections. The pigs examined by Harms *et al.* (2002) had clinical manifestations of respiratory disease and therefore the lesions observed in that study were more severe than in the present study. The results of the present study show that the histopathology of PRDC is complex as nearly all pulmonary reaction patterns can co-exist. Thickened alveolar septa were observed in pigs from both control and case groups, indicating that this finding is not entirely related to bronchopneumonia. Thus, the lesion could be regarded as a pulmonary response to environmental factors associated with swine production, e.g. dust and ammonia. Furthermore, thickening of alveolar septa was not associated with the presence of specific pathogens. Type II hyperplasia occurred more frequently in cases of CBP compared to controls. However, the lesion does not appear to be associated with the age of disease, since it was present in all diagnosis groups. Besides the bronchiolar polyps in the pigs with pneumonic lesions, we also found structures that resembled alveolar polyps, i.e. they protruded from the mucous membrane in the alveoli and were covered by epithelium. Three different forms of alveolar polyp-like structures were identified: fibrinous, cell infiltrated fibrinous, and collagenous, probably representing different ages of the same initial lesion. Alveolar polyp-like structures have not been described before, and their significance is obscure.

Most authors define PRDC as a multifactorial respiratory disease involving several pathogens (Fachinger *et al.*, 2008; Harms *et al.*, 2002; Kim *et al.*, 2003; Opriessnig *et al.*, 2007), whereas Thacker (2006) specifies PRDC as enzootic pneumonia, i.e. infection with *Mycoplasma* spp. and opportunistic bacteria, aggravated by respiratory viruses. Except for 14 animals, *Mycoplasma* spp., bacteria and respiratory viruses were identified in all cases of CBP. This pathogen profile, combined with the pathological findings is in accordance with the diagnosis of PRDC, though we cannot predict the order of infection. Based on the pathogen combination, we only found "classic" enzootic pneumonia in 5 animals with CBP, which indicates that PRDC is more prevalent in Danish finishing pigs, compared to enzootic pneumonia. This probably corresponds to the findings in other countries, where a mixture of mycoplasmas, bacteria and viruses are identified in the majority of porcine pneumonias (Harms *et al.*, 2002; Kim *et al.*, 2003; Palzer *et al.*, 2007).

Twelve different pathogens were obtained from the lungs, and the spectrum of pathogens correlated with observations in other countries (Chiou *et al.*, 2004; Palzer *et al.*, 2008). The following potential pathogens (Halbur, 1998; Liljegren *et al.*, 2003; Stevenson, 1998) were only present in the case group: *S. aureus*, [*H.*] *parasuis*, *A. pleuropneumonia* and PRRSV (EU and US type). Therefore, these agents might represent more aggressive pathogens in cases of PRDC, although the difference between cases and controls was not significant. The diversity and number of pathogens were higher among the diseased animals compared to the control group, supporting the definition of PRDC as a multifactorial disease (Harms *et al.*, 2002), which should be considered when diagnosing and treating cases of PRDC, and in prophylactic strategies. The observed tendency of a lower number of pathogens in the chronic cases of CBP (figure 3), compared to the acute and subacute cases, could be explained by regression of the infection and elimination of pathogens.

The pathogens detected most frequently from the PRDC affected swine were PCV2, *M. hyopneumoniae*, *M. hyorhinis* and *P. multocida*. These pathogens were also found, but in different proportions, from pigs with pneumonia in Germany (Palzer *et al.*, 2008), Taiwan (Chiou *et al.*, 2004) and in the US (Choi *et al.*, 2003). The divergence in ratio of pathogens among these studies depends on the identification methods used, differences in health status of the animals, management factors of the farms, seasonal variation or it could be an indication of the complex nature of pneumonia in swine (Sørensen *et al.*, 2006).

In agreement with other studies (Chiou *et al.*, 2004; Palzer *et al.*, 2008), except results from the US (Choi *et al.*, 2003), we detected PCV2 from the majority of the lung samples. The mere identification of PCV2 by PCR is not regarded as diagnostic for PCV2 associated disease, i.e. porcine circovirus diseases (PCVDs), therefore the animals in the present study can be subclinically infected (Opriessnig *et al.*, 2007). PCV2 has the potential to reduce acquired immunity to other pathogens (Opriessnig *et al.*, 2006). Likewise, Fachinger *et al.* (2008) reported that vaccination against PCV2 reduced co-infections by secondary pathogens and lowered the incidence of pulmonary co-infections. In recent years it has been speculated if PCV2 plays a part in PRDC and this is now widely accepted (Allan and McNeilly, 2006; Harms *et al.*, 2002; Thacker, 2006). In our study, PCV2 was part of the majority of significant associations between pairs of pathogens in the cases of CBP (table 4). Except for *M. hyopneumoniae*, PCV2 and the associated pathogens had a positive influence on each other, i.e. the presence of one increased the chance of finding the other. Although we also detected PCV2 in most control animals, only 1 significant association between PCV2 and other pathogens was observed in this group. These findings indicate that PCV2 could be involved in PRDC in Danish finishing pigs, preparing the way for other respiratory pathogens or occurring as a secondary invader.

M. hyopneumoniae was significantly more prevalent in cases of CBP compared to controls. It is an important pathogen in porcine pneumonia, able to cause disease on its own and in combination with other pathogens (Sørensen *et al.*, 1997; Thacker, 2004). Co-occurrence was seen between *M. hyopneumoniae* and PRRSV or *S. suis*, which is in accordance with other reports on pathogen interaction in respiratory disease (Thacker, 2001; Thacker, 2004). The negative association found between *M. hyopneumoniae* and PCV2 is obscure. Recent studies indicate that *M. hyorhinis* also is important in enzootic pneumonia and respiratory disease in general (Falk *et al.*, 1991; Kawashima *et al.*, 1996; Lin *et al.*, 2006). In the present study, the isolation of *M. hyorhinis* in a significantly higher frequency from the CBP affected pigs, indicates that this pathogen does have an effect on PRDC development in Danish swine.

Confirming previous results (Falk *et al.*, 1991; Gois *et al.*, 1975), *P. multocida* was isolated in approximately 80% of the cases of CBP, which was significantly higher than in the control group. Furthermore, *P. multocida* was found with increased frequency in some subacute (SSBP, SMBP) and chronic (CNBP) groups, compared with other diagnosis groups. Furthermore, *P. multocida* was

significantly associated with histological lesions. This indicates that *P. multocida* most likely is a secondary invading pathogen and aggravates the disease in cases of CBP, which is in agreement with the literature (Morrison *et al.*, 1985; Hoie *et al.*, 1991; Ross, 2006). The finding of *P. multocida* being associated with necrotic lesions has also been reported in both naturally and experimentally infected pigs (Berndt *et al.*, 2002; Ono *et al.*, 2003; Pijoan, 2006). In this study, we observed a significant co-occurrence between *P. multocida* and *M. hyorhinis* in natural cases of CBP, which is in accordance with field studies by Falk *et al.* (1991).

S. suis is a natural inhabitant of the respiratory tract, but it is also associated with suppurative bronchopneumonia, however if it is a primary or secondary pathogen is still to be disclosed (Higgins and Gottschalk, 2006; Thacker, 2001). Although not significant, we isolated *S. suis* from a higher proportion of the PRDC affected animals (45%), than of the control animals (13%), suggesting that *S. suis* is involved in PRDC. *S. suis* was isolated from significantly fewer acute cases (ASBP), compared to the subacute and chronic cases, indicating that it is a secondary invading pathogen. Furthermore, co-occurrence was detected between *S. suis* and *M. hyopneumoniae* or PCV2, which supports the role of *S. suis* as an opportunistic pathogen.

PCMV mainly causes rhinitis and pneumonia in nursery pigs, but has been associated with respiratory disease in pigs of other ages (Orr *et al.*, 1988; Yoon *et al.*, 1998). In the present study, PCMV was the only pathogen isolated at a higher frequency from the control animals (43%), compared to the PRDC affected animals (24%), but a significant association between PCMV and PCV2 was only found in the cases of PRDC. Among the pathogen combinations, combination no. 3 (table 5) was seen more frequently in the acute cases of CBP, than in the subacute and chronic cases. This combination only differed from combination no. 1 and 4 by the presence of PCMV, thus it can be speculated that presence of PCMV causes exacerbation of the disease. To our knowledge PCMV has not been associated with cases of PRDC before, but the role of PCMV in PRDC is controversial and needs to be elucidated.

In conclusion, PRDC occurs more frequently than enzootic pneumonia among Danish finishing pigs. The previously reported pathogen profile and multifactorial aetiology of PRDC are confirmed. Furthermore, PCV2, *S. suis* and *M. hyorhinis* are associated with PRDC, and *S. suis* and *P. multocida* are involved mainly as secondary pathogens. The role of PCMV in PRDC should be

further elucidated. The histopathology of PRDC is complex and nearly all pulmonary reaction patterns can be observed in combination.

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References

Allan, G. and McNeilly, F. (2006). PMWS/PCVD: Diagnose, disease, and control: What do we know? In: *Proceedings of the 19th International Pig Veterinary Society Congress*, Copenhagen, Denmark, pp. 1-9.

Barrow, G. I. and Feltham, R. K. A. (1993). *Cowan and Steel's manual for the identification of medical bacteria*, 3th Edit., Cambridge University Press.

Berndt, A., Heller, M. and Kosmehl, H. (2002). Cytokine mRNA expression in experimental porcine pneumonia. *Deutsche Tierarztliche Wochenschrift*, **109**, 205-209.

Bochsler, P. N. and Slauson, D. O. (2002). Inflammation and Repair of Tissue. In: *Mechanisms of Disease. A Textbook of Comparative General Pathology*, 3th Edit., D. O. Slauson and B. J. Cooper, Eds., Mosby, Inc., St. Louis, Missouri, USA, pp. 140-245.

Caswell, J. L. and Williams, K. J. (2007). Respiratory system. In: *Jubb, Kennedy, and Palmer's pathology of domestic animals*, 5th Edit., M. G. Maxie, Ed., Saunders Elsevier, Philadelphia, USA, pp. 523-653.

Chadfield, M. S., Christensen, J. P., Christensen, H. and Bisgaard, M. (2004). Characterization of streptococci and enterococci associated with septicaemia in broiler parents with a high prevalence of endocarditis. *Avian Pathology*, **33**, 610-617.

Chiou, M. T., Lin, C. N., Chuang, M. S., Chiang, H. C. and Tsai, C. P. (2004). Etiological and epidemiological survey of PRDC associated pathogens in Taiwan. In: *Proceedings of the 18th International Pig Veterinary Society Congress*, Hamburg, Germany, p. 191.

Choi, Y. K., Goyal, S. M. and Joo, H. S. (2003). Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. *Canadian Veterinary Journal*, **44**, 735-737.

Christensen, G. and Enoe, C. (1999). The prevalence of pneumonia, pleuritis, pericarditis and liver spots in Danish slaughter pigs in 1998, including comparation with 1994. *Dansk Veterinaertidsskrift*, **82**, 1006-1015.

Davies, P. R., Moore, M. J. and Pointon, A. M. (1992). The association between lesions of pleuritis and enzootic pneumonia in slaughtered pigs. In: *Proceedings of the 12th International Pig Veterinary Society Congress*, Hague, Netherlands, p. 306.

Fachinger, V., Bischoff, R., Jedidia, S. B., Saalmuller, A. and Elbers, K. (2008). The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. *Vaccine*, **26**, 1488-1499.

Falk, K., Hoie, S. and Lium, B. M. (1991). An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. II. Enzootic pneumonia of pigs: microbiological findings and their relationship to pathomorphology. *Acta Veterinaria Scandinavica*, **32**, 67-77.

Gois, M., Sisak, F., Kuksa, F. and Sovadina, M. (1975). Incidence and evaluation of the microbial flora in the lungs of pigs with enzootic pneumonia. *Zentralblatt fur Veterinarmedizin.Reihe B*, **22**, 205-219.

Grest, P., Keller, H., Sydler, T. and Pospischil, A. (1997). The prevalence of lung lesions in pigs at slaughter in Switzerland. *Schweizer Archiv fur Tierheilkunde*, **139**, 500-506.

Halbur, G. H. (1998). Porcine Viral Respiratory Diseases. In: *Proceedings of the 15th International Pig Veterinary Society Congress*, Birmingham, England, pp. 1-9.

Harder, T. C. and Huebert, P. (2004). Development and application of a nonaplex RT-PCR for simultaneous detection of mycoplasmal and viral agents associated with the porcine respiratory disease complex. In: *Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany*, p. 341.

Harding, J. C. and Halbur, P. G. (2002). PMWS or a Group of PCV2-Associated Syndromes: Ever-Growing Concerns. In: *Proceedings of the 17th International Pig Veterinary Society Congress. PMWS and PCV2 Diseases, Beyond the Debate, Keynotes on the Merial Symposium and Brief Epidemiological Updates*, Ames, Iowa, USA, pp. 19-31.

Harms, P. A., Halbur, P. G. and Sorden, S. D. (2002). Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. *Journal of Swine Health and Production*, **10**, 27-30.

Higgins, R. and Gottschalk, M. (2006). Streptococcal Diseases. In: *Diseases of Swine*, 9th Edit., B. E. Straw, J. J. Zimmerman, S. D'Allaire and D. J. Taylor, Eds., Blackwell Publishing, Oxford, pp. 769-783.

Hoie, S., Falk, K. and Lium, B. M. (1991). An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. IV. Bacteriological findings in chronic pneumonic lesions. *Acta Veterinaria Scandinavica*, **32**, 395-402.

Kawashima, K., Yamada, S., Kobayashi, H. and Narita, M. (1996). Detection of porcine reproductive and respiratory syndrome virus and *Mycoplasma hyorhinis* antigens in pulmonary lesions of pigs suffering from respiratory distress. *Journal of Comparative Pathology*, **114**, 315-323.

Kim, J., Chung, H. K. and Chae, C. (2003). Association of porcine circovirus 2 with porcine respiratory disease complex. *Veterinary Journal*, **166**, 251-256.

Liljegren, C. H., Aalbaek, B., Nielsen, O. L. and Jensen, H. E. (2003). Some new aspects of the pathology, pathogenesis, and aetiology of disseminated lung lesions in slaughter pigs. *APMIS*, **111**, 531-538.

Lin, J. H., Chen, S. P., Yeh, K. S. and Weng, C. N. (2006). *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. *Veterinary Microbiology*, **115**, 111-116.

Luna, L. G. (1968). *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3th Edit., McGraw-Hill Book Company, New York.

Maes, D. G., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., Vrijens, B., Ducatelle, R. and De, K. A. (2001). Non-infectious factors associated with macroscopic and microscopic lung lesions in slaughter pigs from farrow-to-finish herds. *Veterinary Record*, **148**, 41-46.

Morrison, R.B., Pijoan,C., Hilley,H.D. and Rapp,V. (1985). Microorganisms associated with pneumonia in slaughter weight swine. *Canadian Journal of Comparative Medicine,***49**, 129-137.

Ono, M., Okada, M., Namimatsu, T., Fujii, S., Mukai, T. and Sakano, T. (2003). Septicaemia and arthritis in pigs experimentally infected with *Pasteurella multocida* capsular serotype A. *Journal of Comparative Pathology*, **129**, 251-258.

Opriessnig, T., McKeown, N. E., Harmon, K. L., Meng, X. J. and Halbur, P. G. (2006). Porcine circovirus type 2 infection decreases the efficacy of a modified live porcine reproductive and respiratory syndrome virus vaccine. *Clinical and Vaccine Immunology*, **13**, 923-929.

Opriessnig, T., Meng, X. J. and Halbur, P. G. (2007). Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *Journal of Veterinary Diagnostic Investigation*, **19**, 591-615.

Orr, J. P., Althouse, E., Dulac, G. C. and Durham, P. J. (1988). Epizootic Infection of a Minimal Disease Swine Herd with a Herpesvirus. *Canadian Veterinary Journal*, **29**, 45-50.

Osborne, A. D., Saunders, J. R. and Sebunya, T. (1981). An abattoir survey of the incidence of pneumonia in Saskatchewan swine and an investigation of the microbiology of affected lungs. *Canadian Veterinary Journal*, **22**, 82-85.

Palzer, A., Ritzmann, M., Majzoub, M., Wolf, G., Hermanns, W. and Heinritzi, K. (2007). [Frequency of occurrence of pneumonia associated agents and their correlation with clinical and pathological-anatomical findings in pigs]. *Berliner und Munchener Tierarztliche Wochenschrift*, **120**, 483-489.

Palzer, A., Ritzmann, M., Wolf, G. and Heinritzi, K. (2008). Associations between pathogens in healthy pigs and pigs with pneumonia. *Veterinary Record*, **162**, 267-271.

Pijoan, C. (2006). Pneumonic pasteurellosis. In: *Diseases in Swine*, 9th Edit., B. E. Straw, J. J. Zimmerman, S. D'Allaire and D. J. Taylor, Eds., Iowa State University Press, Ames, IA, pp. 719-726.

Plopper, C. G. and Adams, D. R. (2006). Respiratory system. In: *Dellmann's textbook of veterinary histology*, 6th Edit., J. A. Eurell and B. L. Frappier, Eds., Blackwell Publishing Professional, Iowa, USA, pp. 153-169.

Ross, R. F. (1999). Mycoplasmal Diseases. In: *Diseases of Swine*, 8th Edit., B. E. Straw, S. D'Allaire, W. L. Mengeling and D. J. Taylor, Eds., Blackwell Science, Oxford, pp. 495-509.

Ross, R. F. (2006). *Pasteurella multocida* and its role in porcine pneumonia. *Animal Health Research Reviews*, **7**, 13-29.

Soerensen, C. M., Holmskov, U., Aalbaek, B., Boye, M., Heegaard, P. M. and Nielsen, O. L. (2005). Pulmonary infections in swine induce altered porcine surfactant protein D expression and localization to dendritic cells in bronchial-associated lymphoid tissue. *Immunology*, **115**, 526-535.

Sørensen, V., Ahrens, P., Barfod, K., Feenstra, A. A., Feld, N. C., Friis, N. F., Bille-Hansen, V., Jensen, N. E. and Pedersen, M. W. (1997). *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four diagnostic assays. *Veterinary Microbiology*, **54**, 23-34.

Sørensen, V., Jorsal, S. E. and Mousing, J. (2006). Diseases of the Respiratory System. In: *Diseases of Swine*, 9th Edit., B. E. Straw, J. J. Zimmerman, S. D'Allaire and D. J. Taylor, Eds., Blackwell Publishing, Oxford, pp. 149-177.

Stevens, A. and Wilson, I. G. (1996). The haematoxylins and eosin. In: *Theory and practice of Histological Techniques*, 4th Edit., J. D. Bancroft and A. Stevens, Eds., Churchill Livingstone, New York, pp. 99-112.

Stevenson, G. W. (1998). Bacterial pneumonia in swine. In: *Proceedings of the 15th International Pig Veterinary Society Congress*, Birmingham, England, pp. 11-20.

Thacker, E. L. (2001). Immunology of the porcine respiratory disease complex. *Veterinary Clinics of North America.Food Animal Practice*, **17**, 551-565.

Thacker, E. L. (2004). Diagnosis of *Mycoplasma hyopneumoniae*. *Animal Health Research Reviews*, **5**, 317-320.

Thacker, E. L. (2006). Mycoplasmal Diseases. In: *Diseases of Swine*, 9th Edit., B. E. Straw, J. J. Zimmerman, S. D'Allaire and D. J. Taylor, Eds., Blackwell Publishing, Oxford, pp. 701-717.

Wilson, M. R., Takov, R., Friendship, R. M., Martin, S. W., McMillan, I., Hacker, R. R. and Swaminathan, S. (1986). Prevalence of respiratory diseases and their association with growth rate and space in randomly selected swine herds. *Canadian Journal of Veterinary Research*, **50**, 209-216.

Yoon, K. J., Anderson, M. S. and Zimmerman, J. J. (1998). Assessing the significance of porcine cytomegalovirus in respiratory disease of young swine. In: *Proceedings of the 15th International Pig Veterinary Society Congress*, Birmingham, England, p. 173.

Tables

Table 1
Distribution of histopathological diagnoses (definitive diagnoses) of 148 cases of cranioventral lobular bronchopneumonia (BP) in pigs

Lesion age	*n*	*%*	*Exudate*	*n*	*%*	*Sub group*	*n*	*%*
Acute	10	7						
			Suppurative BP (ASBP)	10	100			
Subacute	24	16						
			Suppurative BP (SSBP)	16	67			
						Suppurative	15	94
						Suppurative necrotizing	1	6
			Mixed BP (SMBP)	6	25			
			Non-suppurative BP (SNBP)	2	8			
Chronic	114	77						
			Suppurative BP (CSBP)	52	46			
						Suppurative	44	85
						Suppurative necrotizing	7	13
						Suppurative fibrotic	1	2
			Mixed BP (CMBP)	37	32			
						Mixed	34	92
						Mixed necrotizing	1	3
						Mixed fibrotic	2	5
			Non-suppurative BP (CNBP)	44	22			
						Non-suppurative	7	84
						Non-suppurative fibrotic	1	16

Table 2
Distribution of major histopathologic findings in the lungs from control animals and cases of cranioventral bronchopneumonia (CBP)

		Control		*CBP**					
		(n = 60)		*Acute (n = 10)*		*Subacute (n = 24)*		*Chronic (n = 114)*	
		n	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Bronchitis		1	2	3	30	3	12	14	12
Bronchiolitis		1	2	6	60	8	33	30	26
Bronchiolar polyps		0	-	0	-	2	8	21	18
BALT hyperplasia[†]									
	0	41	68	4	40	2	8	14	12
	+	15	25	6	60	4	17	26	23
	++	4	7	0	-	16	67	52	46
	+++	0	-	0	-	0	-	12	11
	++++	0	-	0	-	2	8	10	9
Alveolar oedema		1	2	8	80	18	75	67	59
Fibrin exudation into alveoli		0	-	5	50	7	29	10	9
Type II hyperplasia[‡]		3	5	3	30	7	29	40	35
Thickening of alveolar septa		16	27	1	10	8	33	28	25
Alveolar polyp-like structures		0	-	2	20	9	38	30	26
Smooth-muscle hyperplasia		2	3	1	10	3	13	31	27
Interstitial oedema		2	3	7	70	10	42	34	30
Interstitial fibroplasia		2	3	1	10	5	21	34	30
Necrotic foci		0	-	0	-	1	4	8	7

[*] Cases of CBP were grouped as acute, subacute or chronic based on the histological evaluation
[†] Hyperplasia of the bronchus-associated lymphoid tissue (BALT) was scored: absent (0); mild (+); moderate (++); marked (+++); or extensive (++++), (see text for details)
[‡] Type II hyperplasia = hyperplasia of type II pneumocytes

Table 3
Results of bacteriological examinations and multiplex PCR for viruses and mycoplasmas

	Control animals (n = 60)		*Pigs with CBP* (n = 148)*		*P-value*[†]
	n	*%*	*n*	*%*	
Bacterial culture					
P. multocida[‡]					
+	0	-	17	11	0.004
++	1	2	21	14	0.004
+++	1	2	76	51	<0.001
S. suis	6	10	27	45	NS[§]
S. aureus	0	-	1	1	NS
[*H.*] *parasuis*	0	-	5	3	NS
A. pleuropneumoniae	0	-	2	1	NS
Mixed culture	27	45	6	4	<0.001
Sterile	25	42	12	8	<0.001
Multiplex PCR					
PCV2[ı]	56	93	143	97	NS
PRRSV[#]					
(EU type)	0	-	8	5	NS
(US type)	0	-	1	1	NS
PCMV[¶]	26	43	35	24	NS
PRCV[**]	0	-	0	-	-
SIV-A[††]	1	2	3	2	NS
M. hyopneumoniae	21	37	142	96	<0.001
M. hyorhinis	22	35	118	80	<0.001

[*] CBP = cranioventral bronchopneumonia
[†] P-values for differences between cases and controls were calculated by either the Chi-square or Fischer's exact test as appropriate
[‡] Growth of *P. multocida* was graded as follows; weak growth on the plate (+), dense growth (++) or massive growth (+++)
[§] NS = not significant
[ı] PCV2 = porcine circovirus type 2
[#] PRRSV = porcine reproductive and respiratory syndrome virus; EU type = European type; US type = North American type
[¶] PCMV = porcine cytomegalovirus
[**] PRCV = porcine respiratory coronavirus
[††] SIV-A = swine influenza virus type A

Table 4
Significant associations between pairs of pathogens among cases of cranioventral bronchopneumonia (n=148) and control animals (n=60)

Associated pathogens	*P-value**	*Spearman-rho factor*
Animals with CBP		
PCV2[†] & *P. multocida* (+)[‡]	0.017	0.067
PCV2 & *S. suis*	0.021	0.088
PCV2 & *M. hyopneumoniae*	0.012	-0.038
PCV2 & PRRSV[§]	0.013	0.048
PCV2 & PCMV[ǀ]	0.025	0.104
P. multocida (++) & *M. hyorhinis*	0.050	0.157
S. suis & *M. hyopneumoniae*	0.022	0.097
PRRSV & *M. hyopneumoniae*	0.014	0.052
Control animals		
PCV2 & *S. suis*	0.030	0.097

*P-values were tested by the Fischer's exact test and the degree of association was calculated by the Spearman-rho factor-test
†PCV2 = porcine circovirus type 2
‡Weak growth of *P. multocida* on the plate (+)
§PRRSV = porcine reproductive and respiratory syndrome virus
ǀPCMV = porcine cytomegalovirus

Table 5
The 10 most frequent combinations of pathogens among cases of cranioventral bronchopneumonia (CBP) and control animals

	Combination number									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Mixed culture							x			
P. multocida[*] +										x
P. multocida[†] ++				x						
P. multocida[‡] +++	x		x			x				
S. suis		x								
PCMV[§]			x		x				x	
PCV2[ǀ]	x	x	x	x	x	x	x	x	x	x
M. hyopneumoniae	x	x	x	x		x			x	x
M. hyorhinis	x	x	x	x					x	x
Frequency of combination										
CBP										
Acute	3	-	3[#]	1	-	1	-	-	-	-
Subacute	4	1	3	3	-	-	-	-	-	1
Chronic	33	9	8	6	1	8	-	-	3	4
Controls	-	4	1	1	8	-	9	7	2	-

*Weak growth of *P. multocida* on the plate (+)
†Dense growth of *P. multocida* on the plate (++)
‡Massive growth of *P. multocida* on the plate (+++)
§PCMV = porcine cytomegalovirus
ǀPCV2 = porcine circovirus type 2
#Significant association

Table 6
Results from bacterial cultivation and multiplex PCR for viruses and mycoplasmas. Numbers and percentage of positive animals are listed for the 148 pigs with cranioventral bronchopneumonia (CBP) in relation to diagnosis

	Acute		*Subacute*						*Chronic*					
	ASBP (n=10)*		*SSBP* (n=16)*		*SMBP* (n=6)*		*SNBP* (n=2)*		*CSBP* (n=52)*		*CMBP* (n=37)*		*CNBP* (n=25)*	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Bacterial culture														
P. multocida[†]														
+	0	-	2	13	1	17	0	-	4	8	4	11	6[‡]	24
++	2	20	5[‡]	31	3[‡]	50	1	50	5	10	2	5	3	12
+++	7	70	8	50	1	17	1	50	31	60	18	49	10	40
S. suis	0[‡]	-	4	25	2	33	0	-	7	13	8	22	6	24
S. aureus	0	-	0	-	0	-	1	50	0	-	0	-	0	-
[*H.*] *parasuis*	0	-	0	-	0	-	0	-	2	4	2	5	1	4
A. pleuropneumoniae	0	-	0	-	0	-	0	-	1	2	0	-	0	-
Mixed culture	2	20	0	-	0	-	0	-	0	-	4	11	0	-
Sterile	0	-	0	-	0	-	0	-	5	10	3	8	4	16
Multiplex PCR														
PCV2[§]	10	100	14	88	6	100	2	100	50	96	37	100	24	96
PRRSV[ǀ]														
(EU type)	1	10	3	19	0	-	0	-	2	4	1	3	1	4
(US type)	0	-	0	-	0	-	0	-	1	2	0	-	0	-
PCMV[#]	3	30	3	19	1	17	0	-	15	29	7	19	6	24
SIV-A[¶]	0	-	0	-	0	-	0	-	0	-	2	5	1	4
M. hyopneumoniae	10	100	15	94	6	100	1	50	51	98	35	95	24	96
M. hyorhinis	9	90	13	81	6	100	2	100	41	79	30	81	17	68

*Compare to table 1

[†]Growth of *P. multocida* was graded as follows; weak growth on the plate (+), dense growth (++) or massive growth (+++)

[‡] The frequency of each pathogen, detected in the diagnosis group, was tested against the remaining cases of CBP by the Chi-square or Fischer's exact test. Significant associations are marked by [‡]

[§]PCV2 = porcine circovirus type 2

[ǀ]PRRSV = porcine reproductive and respiratory syndrome virus; EU type = European type; US type = North American type

[#]PCMV = porcine cytomegalovirus

[¶]SIV-A = swine influenza virus type A

Figure legends

Fig. 1. Porcine lung tissue with different histological lesions found in cases of cranioventral bronchopneumonia

(a). Mild BALT hyperplasia (+), i.e. diffuse infiltration of lymphocytes (arrows) in the peribronchial, peribronchiolar and perivascular tissues including the lamina propria of the airways. Bronchiole (B) and blood vessel (V). HE. Bar, 250 μm.

(b). Moderate BALT hyperplasia (++), i.e. increased diffuse infiltration of lymphocytes and/or presence of a few lymphoid nodules (N). Bronchiole (B). HE. Bar, 250 μm.

(c). Marked BALT hyperplasia (+++), i.e. considerable number of lymphoid nodules (N). Bronchiole (B). HE. Bar, 250 μm.

(d). Extensive BALT hyperplasia (++++), i.e. extensive number of lymphoid nodules (N) affecting most of the lung section. Compressed bronchiole (B) can be seen. HE. Bar, 250 μm.

(e). Suppurative exudate: cellular infiltrate dominated by neutrophils, acute suppurative bronchopneumonia (ASBP). HE. Bar, 25 μm.

(f). Non-suppurative exudate: cellular infiltrate dominated by mononuclear inflammatory cells, chronic non-suppurative bronchopneumonia (CNBP). HE. Bar, 25 μm.

(g). Mixed exudate: cellular infiltrate with both neutrophils and mononuclear cells, subacute mixed bronchopneumonia (SMBP). HE. Bar, 25 μm.

(h). Marked hyperplasia of type II pneumocytes. More than 50% of the alveolar surface is lined by type II pneumocytes. Immunohistochemical staining for cytokeratin. Bar, 25 μm.

Fig. 2. Porcine lung tissue with different lesions

(a). Gross appearance of chronic cranioventral bronchopneumonia. Insert: lung tissue with bronchial pattern of pulmonary consolidation.

(b). Suppurative bronchiolitis and concurrent epithelial hyperplasia (E). HE. Bar, 25 µm.

(c). Oedematous flooding in alveoli. HE. Bar, 50 µm.

(d). Thickening of alveolar septa consisting of collagenous (blue) tissue. Masson Trichrome. Bar, 50 µm.

(e). Alveolar polyp-like structures, P1 with a fibrinous core and P2 with cellular infiltrate. Both covered by type I and type II pneumocytes, demonstrated by immunohistochemical staining for cytokeratin. Bar, 25 µm.

(f). Alveolar polyp-like structure (P) with a collagenous core (blue). Masson trichrome. Bar, 15 µm.

(g). Focal necrotic encapsulated tissue with central mineralization (M). HE. Bar, 100 µm.

(h). Lightly thickening of alveolar septa from a pig in the control group. HE. Bar, 50 µm.

Fig. 3. The frequency of pathogen count detected in control animals (n=60), and in animals with cranioventral bronchopneumonia; acute (n=10), subacute (n=24) or chronic (n=114).

Figure 1

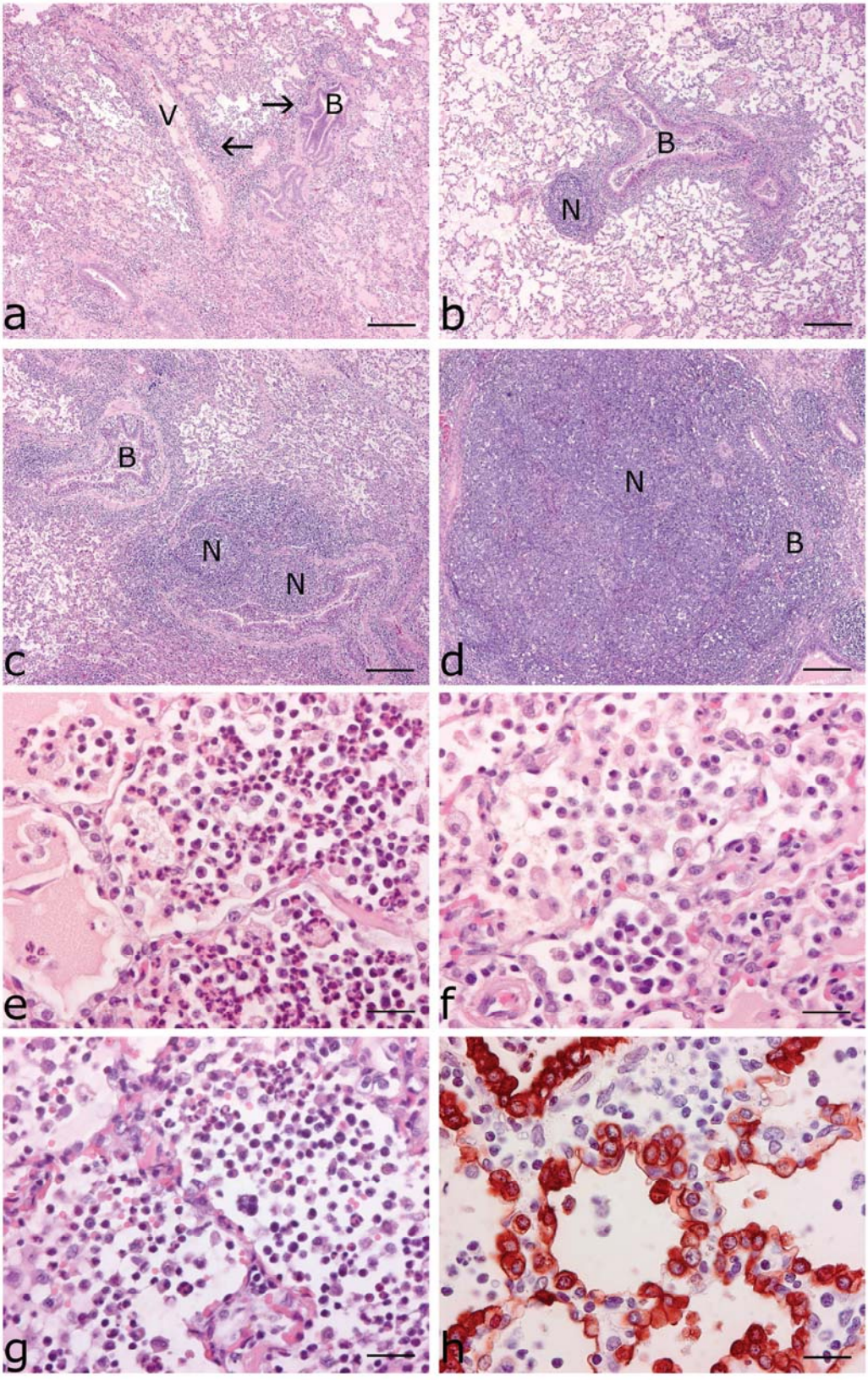


Figure 2

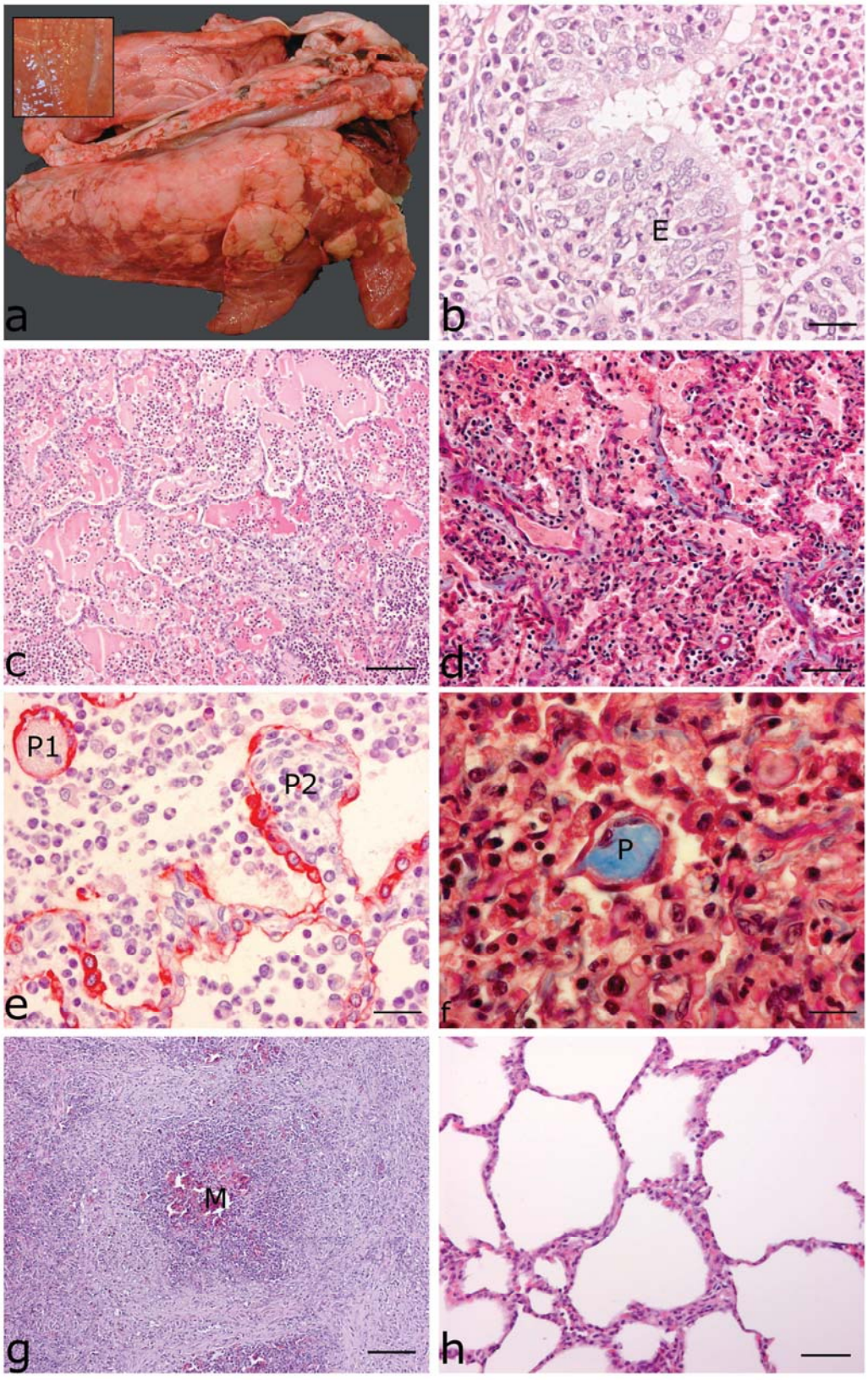
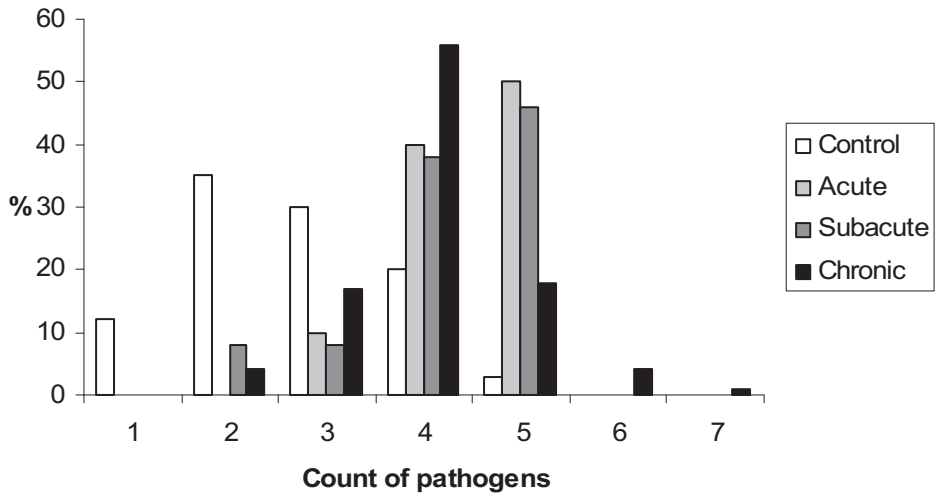


Figure 3


60
50
40
%30
20
10
0
1
2
3
4
5
6
7
Count of pathogens
Control
Acute
Subacute
Chronic

Chapter 4 Discussion and perspectives

The main focus of the present thesis was to look into aspects of the pathogenesis and pathology of experimentally induced and naturally occurring PCVDs, studied by morphological methods, in pigs of different ages.

In manuscript I, an attempt to study the initial pathogenesis was carried out by examining bone marrow and thymus from piglets experimentally inoculated with PCV2. However, no PCV2 or viral replication was detected by ISH in these tissues at 5, 8 or 29 days PI. Another attempt to study the initial pathogenesis in vivo, was carried out by local intestinal PCV2 inoculation of snatch-farrowed colostrum-deprived piglets (data not shown). The local intestinal inoculation experiment (loop-model) was planned by a Dutch, research group at Wageningen University (Stockhofe-Zurwieden et al., 2008), and the experiment itself was performed in collaboration with us. In this study, PCV2 was inoculated directly into the lumen of ligated small intestinal loops. After 2 hours the ligations were removed and the pigs were followed for 44 hours, before euthanization and tissue sampling. PCV2 could not be detected by IHC in any of the harvested tissues (e.g. intestine, regional lymph nodes, spleen or bone marrow). PCV2 was found by PCR in mucosal scrapings, but replication (detected by measuring *cap* mRNA by PCR) could not be detected in these scrapings (Stockhofe-Zurwieden et al., 2008).

The pathogenesis of naturally occurring PCV2 infections (manuscript I and III) was studied by evaluation of PCV2 loads in primary lymphoid organs and by examination for cells permissive of replication. In stillborn and mummified foetuses with PCV2 IHC positive hearts, the virus was also detected in most of the bone marrow samples (thymus was not available), whereas in PMWS-affected pigs with PCV2 in thymus, the virus was only found in half of the bone marrows, and in lower amounts compared to that of the foetuses. In the bone marrow of the PMWS-affected pigs, the detection of PCV2 could not be related to a specific cell line and unfortunately, based on the condition of the material, it was not possible to identify the different cell types in the foetal bone marrow. The difference in PCV2 loads of the foetal and postnatal bone marrows could be related to their developmental stage, as it has been shown that the PCV2 target-cells change during the development from foetal to postnatal life. Thus, in foetuses PCV2 can be found in hepatocytes, histiocytes and myocardiocytes, mainly in the latter, whereas histiocytes are the main target cells in

postnatal life (Sanchez et al., 2003). The foetal tissues and the primary lymphoid organs from the PMWS-affected pigs were also tested for presence of viral replication by ISH. In the foetuses, replication was only detected in low grade in the myocardiocytes, whereas no replication was detected in the bone marrow, liver, lungs or kidneys. In the PMWS-affected pigs, low grade of replication could be seen in both thymus and bone marrow mainly in histiocyte-like cells, but also in cortical lymphocyte-like cells in thymus. This difference in cell types permissive of replication might also represent differences in the developmental stages of the pigs. In both studies, replication was only observed in a few scattered cells and this, even in tissues with high viral loads. The observed discrepancy between the detection of huge amounts of virus in diseased pigs, together with no or low grade of viral replication, is a common finding in other PCV2 replication studies (Hamberg et al. 2007; Pérez-Martín et al. 2007), and it is a fact that has puzzled many. When comparing the number of replication positive samples from the thymus and bone marrow of the PMWS-affected pigs, and the foetal myocardium, to the number of PCV2 positive samples from these organs, it was found that the ratio of replication was similar (31-43%) in the foetal and postnatal tissues. In the postnatal tissues no specific cell type in neither the thymus nor bone marrow seemed to be important in regard to PCV2 replication, corresponding to the findings of previous studies, where no main cell type supporting replication has been identified in other organs (Hamberg et al. 2007; Pérez-Martín et al. 2007; Yu et al. 2007). However, in the foetal tissues we found that PCV2 specifically replicated in myocardiocytes, but only in a low number of cells, whereas replication was not detected in the bone marrow. Since the examined foetal material was somewhat decomposed by the mummification and the bone marrow might be more sensitive to this process, this could explain the negative findings in regard to viral replication. Thus, further studies should be carried out on foetal tissues from experimental settings to elucidate the role of the bone marrow in the foetal PCV2 infection. Based on the role of the primary lymphoid organs in other circovirus infections and the detection of PCV2 in the thymus and bone marrow, it should also be considered to look further into the role of the thymus and ileal Peyer's patch in foetuses, and also examine the ileal Peyer's patch in postnatal pigs.

Another way of studying the pathogenesis of PCV2 infections would be by performing double stainings for replicative forms of PCV2 (detected by ISH) and cellular markers (detected by IHC). This was also attempted on the material in manuscript I (data not shown), but technical problems of combining the ISH and IHC techniques, made this impossible. A way to solve this problem could

be by developing an IHC technique for detection of replicase proteins, which are essential for initiation of the PCV2 replication process and recently, such mAb have been produced and characterized (Zhang et al., 2009).

In manuscript II, PCV2 antigen was detected in association with FDCs in the lymph nodes of finishing pigs and it can be speculated if the persistent viremia reported in PCV2 infection studies (Bolin et al., 2001; Carasova et al., 2007; Darwich et al., 2008), is related to this FDC associated capture of PCV2. Furthermore, as FDCs are intimate with B-lymphocytes, and support and stimulate these cells in different ways, FDCs have the potential to interfere with the functions of B-lymphocytes. Depletion of B-lymphocytes occurs early in the progression of PMWS (Nielsen et al., 2003; Sarli et al., 2001; Segalés et al., 2004a), and apoptosis (Shibahara et al., 2000) or reduced proliferation (Mandrioli et al., 2004; Resendes et al., 2004) has been suggested to be the responsible mechanisms - however this is still controversial. Furthermore, it has been described that PMWS-affected pigs have a reduced production of PCV2 antibodies, especially neutralizing antibodies and therefore, cannot clear the virus efficiently, whereas it seems that pigs that are able to mount a sufficient antibody response only get subclinically infected (Fort et al., 2007; Meerts et al., 2006). Thus, if the PCV2 carrying FDCs have the ability to disturb the development of B-cells into plasma cells, this might cause reduced production of antibodies. This hypothesis might be supported by the discovery and characterization of follicular dendritic cell secreted protein (FDC-SP), which has been isolated from human tonsillar FDCs. FDC-SP is implicated in modulation of B-cell activity, and a negative effect on humoral immunity has been ascribed to this protein (Al-Alwan et al., 2007). Thus, it should be examined if FDCs are responsible for the reduction in number of B-lymphocytes and the reduced humoral immune response of PMWS-affected pigs.

PCV2 antigen was not detected in the nuclei of FDCs, thus it was not suspected that viral replication took place here (manuscript II). However, abundant PCV2 DNA and protein have been detected in the cytoplasm and nucleus of FDCs in 4 weeks old experimentally infected gnotobiotic pigs, including pigs that were only subclinically infected (Krakowka et al., 2002). This suggests that FDCs might also be permissive of replication, at least in gnotobiotes, and thus might be involved in the initial pathogenesis of PCV2 infections and not solely be associated with the persistent infection in older pigs. The exact role of the FDCs in the pathogenesis of PCV2 infections should be elucidated further in experimental settings. This could be examined by in vitro studies, where FDC

cultures were challenged with PCV2 to see how they responded with B-cells afterwards. Another way would be to obtain lymph node biopsies from experimentally infected pigs and hereby, it would be possible in vivo, and in the same animal, to study several stages of the infection. Furthermore, the infective status of the FDC associated PCV2 should be investigated, to see if the virus is still infectious and able to replicate in e.g. PK-15 cells. The subcellular relationship between FDCs, PCV2 and B-lymphocytes could be examined further by performing confocal- and electron microscopical studies.

Serous fat atrophy was detected in the bone marrow of PMWS-affected pigs, and this finding has not been described before (manuscript I). In general the PRDC associated histopathology included almost all possible pulmonary reaction patterns, but no specific reaction was associated with the detection of PCV2 by IHC in the lungs from the cases of PRDC (manuscript II and IV). However, the lung material was obtained from abattoirs, thus if pigs showing clinical manifest PRDC were examined, PCV2 might be demonstrated within the lung lesions. Lymphohistiocytic infiltration is a common lesion of different PCVDs (Opriessnig et al., 2007), which corresponds with the findings of most organs in the present study. Here lymphocytes and/or histiocytes were detected in tissues from foetuses (in the myocardium and perivascular lesions in the liver) (manuscript III), in the kidneys of finishing pigs (manuscript II), in the thymus and bone marrow of PMWS-affected pigs (manuscript I). Thus, in regard to the pathology of PCVDs, it is possible to detect similar lesions in different tissues, of pigs of different ages.

Several methods were used for the detection of PCV2, i.e. ISH (manuscript I), IHC (manuscript II and III) and PCR (manuscript II, III and IV). It was found that PCR was very sensitive and thus, the majority of the lung samples in manuscript IV were found to be positive for PCV2, but when testing the same tissues by IHC the number of positives was reduced (manuscript II). Real-time PCR proved to be the most sensitive method for detection of PCV2 in foetal tissues, as it was only possible to detect PCV2 by IHC early in the outbreak of reproductive failure (manuscript III). Thus, the diagnostic method chosen for specific PCVDs should be considered carefully.

As a curiosity, mature eosinophilic granulocytes and eosinophilic precursor-like cells were detected in the thymus of PMWS-affected pigs (manuscript I). Similar cell types were detected in varying numbers in the lymph node sinus system and red pulp of the spleens from the finishing pigs

(manuscript II). Eosinophilic granulocytes have previously been described as part of the inflammatory infiltrate in lymph nodes, kidneys, gastrointestinal tract and/or liver of pigs experimentally (Ellis et al., 1999; Krakowka et al., 2000) or naturally infected with PCV2 (Nunez et al., 2003). In the present study, the eosinophilic infiltrate was not assumed to be associated with PCV2, as it was not linked to detection of PCV2 in the lymph nodes, spleens or thymus and furthermore, the detection frequencies were equal in the PMWS/PRDC-case and control groups. In the literature, infiltration of eosinophilic granulocytes has been associated with different conditions, like parasitic and viral infections, allergies and cancer (Adamko et al., 2005; Jacobsen et al., 2007). In the present study, no obvious explanation for the observed infiltration of eosinophils could be found, though it can not be ruled out that it was associated with parasitic infiltration.

The observed association between FDCs and PCV2, and the detection of PCV2 in the bone marrow of foetal tissues, might be promising in regard of gaining further knowledge of the pathogenesis of PCVDs.

Chapter 5 Conclusions

Based on the work presented in this thesis, it can be concluded that:

- The primary lymphoid organs are definitively affected by PCV2 infections, but there is no evidence for the thymus or bone marrow being more important than other tissues in regard to PCV2 replication in cases of PMWS.

- In mummified and stillborn foetuses PCV2 was mainly detected in the heart and bone marrow, and viral replication was demonstrated in myocardiocytes.

- PCV2 antigen was found to be associated with FDCs in finishing pigs, but it is not clear whether this represents subclinical or persistent infection, or a status after PCV2 infection with innocent storage of antigen.

- PCV2 antigen could only be detected by IHC in a short window of time early in an outbreak of PCV2 associated reproductive failure.

- In cases of reproductive failure it was found that PCV2 IHC only was suited for diagnosing the acute stages, whereas quantitative PCR proved valuable as a sensitive diagnostic method within a wider time span.

- It was found that measurements of total IgG in foetal pleura cavity fluid not are very reliable as indication of intrauterine infection with PCV2.

- PCV2 antigens were demonstrated by IHC in tissues of 61% of Danish finishing pigs at slaughter, but the findings were not associated with current PCVDs or PCVD-indicative histopathology.

- Significant associations were detected between presence of PCV2 and several other pathogens in cases of PRDC, indicating that PCV2 could be involved in PRDC as a secondary invader or could prepare the way for other respiratory pathogens.

- In cases of PRDC multiple pathogens could be demonstrated, confirming the previously reported pathogen profile and multifactorial aetiology of PRDC.

- The histopathology of PRDC is complex and nearly all pulmonary reaction patterns can be observed in combination.

References

Adamko, D.J., Odemuyiwa, S.O., Vethanayagam, D., Moqbel, R., 2005. The rise of the phoenix: the expanding role of the eosinophil in health and disease. Allergy 60, 13-22.

Al-Alwan, M., Du, Q., Hou, S., Nashed, B., Fan, Y., Yang, X., Marshall, A.J., 2007. Follicular dendritic cell secreted protein (FDC-SP) regulates germinal center and antibody responses. J. Immunol. 178, 7859-7867.

Albina, E., Truong, C., Hutet, E., Blanchard, P., Cariolet, R., L'Hospitalier, R., Mahe, D., Allee, C., Morvan, H., Amenna, N., Le, D.M., Madec, F., Jestin, A., 2001. An experimental model for post-weaning multisystemic wasting syndrome (PMWS) in growing piglets. J. Comp. Pathol. 125, 292-303.

Allan, G.M., Caprioli, A., McNair, I., Lagan-Tregaskis, P., Ellis, J., Krakowka, S., McKillen, J., Ostanello, F., McNeilly, F., 2007. Porcine circovirus 2 replication in colostrum-deprived piglets following experimental infection and immune stimulation using a modified live vaccine against porcine respiratory and reproductive syndrome virus. Zoonoses Public Health 54, 214-222.

Allan, G., McNeilly, F., 2006. PMWS/PCVD: Diagnose, disease, and control: What do we know? In: Proceedings of the 19th International Pig Veterinary Society Congress, Copenhagen, Denmark, pp. 1-9.

Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000a. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. 145, 2421-2429.

Allan, G.M., McNeilly, F., Meehan, B.M., Ellis, J.A., Connor, T.J., McNair, I., Krakowka, S., Kennedy, S., 2000b. A sequential study of experimental infection of pigs with porcine circovirus and porcine parvovirus: immunostaining of cryostat sections and virus isolation. J. Vet. Med. B Infect. Dis. Vet. Public Health 47, 81-94.

Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. 121, 1-11.

Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., 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. Diagn. Invest. 10, 3-10.

Anonymous, 2007. Control of porcine circovirus diseases (PCVDs): Towards improved food quality and safety. [online 22-12-2008]. http://www.pcvd.org/documents/PCVD_newsletter_2007.pdf.

Armstrong, D., Bishop, S.C., 2004. Does genetics or litter effect influence mortality in PMWS. In: Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany, p. 809.

Biagini, P., Todd, D., Bendinelli, M., Hino, S., Mankertz, A., Mishiro, S., Niel, C., Okamoto, H., Raidal, S., Ritchie, B.W., Teo, G.C., 2005. Anellovirus. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 335-341.

Bogdan, J., West, K., Clark, E., Konoby, C., Haines, D., Allan, G., McNeilly, F., Meehan, B., Krakowka, S., Ellis, J.A., 2001. Association of porcine circovirus 2 with reproductive failure in pigs: a retrospective study, 1995-1998. Canadian Veterinary Journal-Revue Veterinaire Canadienne 42, 548-550.

Bolin, S.R., Stoffregen, W.C., Nayar, G.P., Hamel, A.L., 2001. Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-deprived piglets with type 2 porcine circovirus. J. Vet. Diagn. Invest. 13, 185-194.

Brunborg, I.M., Jonassen, C.M., Moldal, T., Bratberg, B., Lium, B., Koenen, F., Schonheit, 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. Invest. 19, 368-375.

Bækbo, P., 2005. PMWS case definition (Herd level). October 2005. In: Sixth framework programme. Priority SSP/5.4.6. Control of Porcine Circovirus Diseases (PCVDs): Towards Improved Food Quality and Safety. Contract no.: 513928

Carasova, P., Celer, V., Takacova, K., Trundova, M., Molinkova, D., Lobova, D., Smola, J., 2007. The levels of PCV2 specific antibodies and viremia in pigs. Res. Vet. Sci. 83, 274-278.

Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lusis, P., Fairles, J., Alexander, H.S., van, D.T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease - 2004-2006. Can. J. Vet. Res. 72, 259-268.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. Vet. J. 169, 326-336.

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. Vet. J. 168, 41-49.

Chang, H.W., Jeng, C.R., Lin, C.M., Liu, J.J., Chang, C.C., Tsai, Y.C., Chia, M.Y., Pang, V.F., 2007. The involvement of Fas/FasL interaction in porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus co-inoculation-associated lymphocyte apoptosis in vitro. Vet. Microbiol. 122, 72-82.

Cheung, A.K., 2007. A stem-loop structure, sequence non-specific, at the origin of DNA replication of porcine circovirus is essential for termination but not for initiation of rolling-circle DNA replication. Virology 363, 229-235.

Cheung, A.K., 2003. The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology 313, 452-459.

Cheung, A.K., Bolin, S.R., 2002. Kinetics of porcine circovirus type 2 replication. Arch. Virol. 147, 43-58.

Chiou, M.T., Lin, C.N., Chuang, M.S., Chiang, H.C., Tsai, C.P., 2004. Etiological and epidemiological survey of PRDC associated pathogens in Taiwan. In: Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany, p. 191.

Choi, C., Chae, C., 2001. Colocalization of porcine reproductive and respiratory syndrome virus and porcine circovirus 2 in porcine dermatitis and nephrology syndrome by double-labeling technique. Vet. Pathol. 38, 436-441.

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.

Choi, Y.K., Goyal, S.M., Joo, H.S., 2003. Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. Can. Vet. J. 44, 735-737.

D'Allaire, S., Moore, C., Cote, G., 2007. A survey on finishing pig mortality associated with porcine circovirus diseases in Quebec. Can. Vet. J. 48, 145-146.

Darwich, L., Segalés, J., Resendes, A., Balasch, M., Plana-Duran, J., Mateu, E., 2008. Transient correlation between viremia levels and IL-10 expression in pigs subclinically infected with porcine circovirus type 2 (PCV2). Res. Vet. Sci. 84, 194-198.

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.

Day, L.A., Hendrix, R.W., 2005. Inoviridae. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 279-287.

Desrosiers, R., 2007. Overview of PCVD - The disease in Eastern Canada and US vs. Europe. In: Proceedings of the 2007 Banff Pork Seminar, Alberta, Canada, pp. 35-48.

Drolet, R., Larochelle, R., Morin, M., Delisle, B., Magar, R., 2003. Detection rates of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, and swine influenza virus in porcine proliferative and necrotizing pneumonia. Vet. Pathol. 40, 143-148.

Drolet, R., Thibault, S., D'Allaire, S., Thomson, J.R., Done, S.H., 1999. Porcine dermatitis and nephropathy syndrome (PDNS): An overview of the disease. J. Swine health prod. 7, 283-285.

Dupont, K., Nielsen, E.O., Baekbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Vet. Microbiol. 128, 56-64.

Ellis, J.A., Allan, G., Krakowka, S., 2008. Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2-associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. Am. J. Vet. Res. 69, 1608-1614.

Ellis, J., Krakowka, S., Lairmore, M., Haines, D., Bratanich, A., Clark, E., Allan, G., Konoby, C., Hassard, L., Meehan, B., Martin, K., Harding, J., Kennedy, S., McNeilly, F., 1999. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J. Vet. Diagn. Invest. 11, 3-14.

Fachinger, V., Bischoff, R., Jedidia, S.B., Saalmuller, A., Elbers, K., 2008. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. Vaccine 26, 1488-1499.

Fane, B., 2005. Microviridae. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 289-299.

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, 541-551.

Fernandes, L.T., Mateu, E., Sibila, M., Fort, M., Andaluz, A., McNeilly, F., Allan, G., Sanchez, A., Segalés, J., Stevenson, L., 2007. Lack of in vitro and in vivo effects of lipopolysaccharide on porcine circovirus type 2 infection. Viral Immunol. 20, 541-552.

Fort, M., Fernandes, L.T., Nofrarias, M., Diaz, I., Sibila, M., Pujols, J., Mateu, E., Segalés, J., 2009. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. Vet. Immunol. Immunopathol. doi: 10.1016/j.vetimm.2008.12.024.

Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F., Segalés, J., 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine 26, 1063-1071.

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, 244-255.

Gauger, P.C., Lager, K.M., Vincent, A.L., Opriessnig, T., Kehrli, M.E., Cheung, A.K., 2008. Pathogenesis of PCV2a and PCV2b virus in germ-free pigs. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, p. 28.

Gilpin, D.F., McCullough, K., Meehan, B.M., McNeilly, F., McNair, I., Stevenson, L.S., Foster, J.C., Ellis, J.A., Krakowka, S., Adair, B.M., Allan, G.M., 2003. In vitro studies on the infection and replication of porcine circovirus type 2 in cells of the porcine immune system. Vet. Immunol. Immunopathol. 94, 149-161.

Grasland, B., Loizel, C., Blanchard, P., Oger, A., Nignol, A.C., Bigarre, L., Morvan, H., Cariolet, R., Jestin, A., 2005. Reproduction of PMWS in immunostimulated SPF piglets transfected with infectious cloned genomic DNA of type 2 porcine circovirus. Vet. Res. 36, 685-697.

Grau-Roma, L., Crisci, E., Sibila, M., Lopez-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segalés, J., 2008a. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet. Microbiol. 128, 23-35.

Grau-Roma, L., Hjulsager, C.K., Sibila, M., Kristensen, C.S., Lopez-Soria, S., Enoe, C., Casal, J., Botner, A., Nofrarias, M., Bille-Hansen, V., Fraile, L., Baekbo, P., Segalés, J., Larsen, L.E., 2008b. 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. S0378-1135(08)00476-8 [pii];10.1016/j.vetmic.2008.10.007 [doi].

Grau-Roma, L., Segalés, J., 2007. Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. Vet. Microbiol. 119, 144-151.

Ha, Y., Lee, Y.H., Ahn, K.K., Kim, B., Chae, C., 2008. Reproduction of postweaning multisystemic wasting syndrome in pigs by prenatal porcine circovirus 2 infection and postnatal porcine parvovirus infection or immunostimulation. Vet. Pathol. 45, 842-848.

Ha, Y., Jung, K., Chae, C., 2005. Lack of evidence of porcine circovirus type 1 and type 2 infection in piglets with congenital tremors in Korea. Vet. Rec. 156, 383-384.

Halbur, G.H., 1998. Porcine Viral Respiratory Diseases. In: Proceedings of the 15th International Pig Veterinary Society Congress, Birmingham, England, pp. 1-9.

Hamberg, A., Ringler, S., Krakowka, S., 2007. A novel method for the detection of porcine circovirus type 2 replicative double stranded viral DNA and nonreplicative single stranded viral DNA in tissue sections. J. Vet. Diagn. Invest. 19, 135-141.

Harding, J.C., Halbur, P.G., 2002. PMWS or a Group of PCV2-Associated Syndromes: Ever-Growing Concerns. In: PMWS and PCV2 Diseases, Beyond the Debate, Keynotes on the Merial Symposium and Brief Epidemiological Updates. The 17th International Pig Veterinary Society Congress, Ames, Iowa, USA, pp. 19-31.

Harding, J.C.S., Clark, E.G., 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). J. Swine health prod. 5, 201-203.

Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. J. Swine Health Prod. 10, 27-30.

Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. 38, 528-539.

Hasslung, F., Wallgren, P., Ladekjaer-Hansen, A.S., Botner, A., Nielsen, J., Wattrang, E., Allan, G.M., McNeilly, F., Ellis, J., Timmusk, S., Belak, K., Segall, T., Melin, L., Berg, M., Fossum, C., 2005. Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. Vet. Microbiol. 106, 49-60.

Hino, S., Miyata, H., 2007. Torque teno virus (TTV): current status. Rev. Med. Virol. 17, 45-57.

Hirai, T., Nunoya, T., Ihara, T., Kusanagi, K., Kato, T., Shibuya, K., 2003. Acute hepatitis in a piglet experimentally inoculated with tissue homogenates from pigs with postweaning multisystemic wasting syndrome. J. Vet. Med. Sci. 65, 1041-1045.

Huang, Y.Y., Walther, I., Martinson, S.A., Lopez, A., Yason, C., Godson, D.L., Clark, E.G., Simko, E., 2008. Porcine circovirus 2 inclusion bodies in pulmonary and renal epithelial cells. Vet. Pathol. 45, 640-644.

Jacobsen, E.A., Taranova, A.G., Lee, N.A., Lee, J.J., 2007. Eosinophils: singularly destructive effector cells or purveyors of immunoregulation? J. Allergy Clin. Immunol. 119, 1313-1320.

Jensen, T.K., Vigre, H., Svensmark, B., Bille-Hansen, V., 2006. Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by Lawsonia intracellularis. J. Comp. Pathol. 135, 176-182.

Johnson, C.S., Joo, H.S., Direksin, K., Yoon, K.J., Choi, Y.K., 2002. Experimental in utero inoculation of late-term swine fetuses with porcine circovirus type 2. J. Vet. Diagn. Invest. 14, 507-512.

Josephson, G., Charbonneau, G., 2001. Case report of reproductive problem in a new startup operation. J. Swine Health Prod. 9, 258-259.

Karuppannan, A.K., Jong, M.H., Lee, S.H., Zhu, Y., Selvaraj, M., Lau, J., Jia, Q., Kwang, J., 2009. Attenuation of porcine circovirus 2 in SPF piglets by abrogation of ORF3 function. Virology 383, 338-347.

Kennedy, S., Segalés, J., Rovira, A., Scholes, S., Domingo, M., Moffett, D., Meehan, B., O'Neill, R., McNeilly, F., Allan, G., 2003. Absence of evidence of porcine circovirus infection in piglets with congenital tremors. J. Vet. Diagn. Invest. 15, 151-156.

Kim, J., Chae, C., 2005. Necrotising lymphadenitis associated with porcine circovirus type 2 in pigs. Vet. Rec. 156, 177-178.

Kim, J., Chae, C., 2004a. A comparison of virus isolation, polymerase chain reaction, immunohistochemistry, and in situ hybridization for the detection of porcine circovirus 2 and porcine parvovirus in experimentally and naturally coinfected pigs. J. Vet. Diagn. Invest. 16, 45-50.

Kim, J., Chae, C., 2004b. Concurrent presence of porcine circovirus type 2 and porcine parvovirus in retrospective cases of exudative epidermitis in pigs. Vet. J. 167, 104-106.

Kim, J., Ha, Y., Jung, K., Choi, C., Chae, C., 2004. Enteritis associated with porcine circovirus 2 in pigs. Can. J. Vet. Res. 68, 218-221.

Kim, J., Chung, H.K., Chae, C., 2003. Association of porcine circovirus 2 with porcine respiratory disease complex. Vet. J. 166, 251-256.

Kolb, J.R., Genzow, M., Roof, M.R., 2006. PCV2 induced granulomatous enteritis in growing pigs. In: Proceedings of the 19th International Pig Veterinary Society Congress, Copenhagen, Denmark, p. 272.

Krakowka, S., Ellis, J., McNeilly, F., Waldner, C., Rings, D.M., Allan, G., 2007. Mycoplasma hyopneumoniae bacterins and porcine circovirus type 2 (PCV2) infection: induction of postweaning multisystemic wasting syndrome (PMWS) in the gnotobiotic swine model of PCV2-associated disease. Can. Vet. J. 48, 716-724.

Krakowka, S., Ellis, J.A., McNeilly, F., Gilpin, D., Meehan, B., McCullough, K., Allan, G., 2002. Immunologic features of porcine circovirus type 2 infection. Viral Immunol. 15, 567-582.

Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. 38, 31-42.

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.

Ladekjaer-Mikkelsen, A.S., Nielsen, J., Stadejek, T., Storgaard, T., Krakowka, S., Ellis, J., McNeilly, F., Allan, G., Botner, A., 2002. Reproduction of postweaning multisystemic wasting syndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old piglets experimentally infected with porcine circovirus type 2 (PCV2). Vet. Microbiol. 89, 97-114.

Ladekjaer-Mikkelsen, A.S., Nielsen, J., Storgaard, T., Botner, A., Allan, G., McNeilly, F., 2001. Transplacental infection with PCV-2 associated with reproductive failure in a gilt. Vet. Rec. 148, 759-760.

Lager, K.M., Gauger, P.C., Vincent, A.L., Opriessnig, T., Kehrli, M.E., Cheung, A.K., 2007. Mortality in pigs given porcine circovirus type 2 subgroup 1 and 2 viruses derived from DNA clones. Vet. Rec. 161, 428-429.

Lainson, F.A., Aitchison, K.D., Donachie, W., Thomson, J.R., 2002. Typing of Pasteurella multocida isolated from pigs with and without porcine dermatitis and nephropathy syndrome. J. Clin. Microbiol. 40, 588-593.

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, 114-120.

Larochelle, R., Sauvageau, R., Magar, R., 1994. Immunohistochemical detection of swine influenza virus and porcine reproductive and respiratory syndrome virus in porcine proliferative and necrotizing pneumonia cases from Quebec. Can. Vet. J. 35, 513-515.

Lefebvre, D., 2009. Genetic, antigenetic and pathogenetic features of porcine circovirus type 2 strains. Ph.D.Thesis, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium.

Lefebvre, D.J., Barbé, F., Atanasova, K., Nauwynck, H.J., 2009. Clinical and virological outcome of experimental PCV2a and PCV2b infections in midgestational porcine foetuses. In: D. Lefebvre (Ed.). Genetic, Antigenetic and pathogenetic features of porcine circovirus type 2 strains. Ph.D. Thesis, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium. pp. 79-95.

Lin, C.M., Jeng, C.R., Chang, H.W., Guo, I.C., Huang, Y.L., Tsai, Y.C., Chia, M.Y., Pang, V.F., 2008. Characterization of porcine circovirus type 2 (PCV2) infection in swine lymphocytes using mitogen-stimulated peripheral blood lymphocytes from healthy PCV2-carrier pigs. Vet. Immunol. Immunopathol. 124, 355-366.

Liu, J., Chen, I., Du, Q., Chua, H., Kwang, J., 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. J. Virol. 80, 5065-5073.

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.

Magar, R., Larochelle, R., Thibault, S., Lamontagne, L., 2000. Experimental transmission of porcine circovirus type 2 (PCV2) in weaned pigs: a sequential study. J. Comp. Pathol. 123, 258-269.

Majzoub, M., Ritzmann, I., Truyen, U., Matiasek, K., Heinritzi, K., Hermanns, W., 2005. Porcine dermatitis nephropathy syndrome (PDNS) - a study of pathomorphologic changes. Tieraerztl. Prax. Ausg. G Grosstiere Nutztiere 33, 169-174.

Maldonado, J., Segalés, J., Martinez-Puig, D., Calsamiglia, M., Riera, P., Domingo, M., Artigas, C., 2005. Identification of viral pathogens in aborted fetuses and stillborn piglets from cases of swine reproductive failure in Spain. Vet. J. 169, 454-456.

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.

Mankertz, A., Caliskan, 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.

Mankertz, J., Buhk, H.J., Blaess, G., Mankertz, A., 1998. Transcription analysis of porcine circovirus (PCV). Virus Genes 16, 267-276.

Mateusen, B., Maes, D.G., Van, S.A., Lefebvre, D., Nauwynck, H.J., 2007. Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. Theriogenology 68, 896-901.

Mateusen, B., Sanchez, R.E., Van, S.A., Meerts, P., Maes, D.G., Nauwynck, H.J., 2004. Susceptibility of pig embryos to porcine circovirus type 2 infection. Theriogenology 61, 91-101.

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., Misinzo, G., Lefebvre, D., Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Vet. Res. doi: 10.1186/1746-6148-2-6.

Meerts, P., Misinzo, G., McNeilly, F., Nauwynck, H.J., 2005a. Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, fetal cardiomyocytes and macrophages. Arch. Virol. 150, 427-441.

Meerts, P., Van Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005b. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. Viral Immunol. 18, 333-341.

Mikami, O., Nakajima, H., Kawashima, K., Yoshii, M., Nakajima, Y., 2005. Nonsuppurative myocarditis caused by porcine circovirus type 2 in a weak-born piglet. J. Vet. Med. Sci. 67, 735-738.

Misinzo, G., Delputte, P.L., Lefebvre, D.J., Nauwynck, H.J., 2009. Porcine circovirus 2 infection of epithelial cells is clathrin-, caveolae- and dynamin-independent, actin and Rho-GTPase-mediated, and enhanced by cholesterol depletion. Virus Res. 139, 1-9.

Misinzo, G., Delputte, P.L., Meerts, P., Lefebvre, D.J., Nauwynck, H.J., 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. J. Virol. 80, 3487-3494.

Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H.M., Nauwynck, H.J., 2005. Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. J. Gen. Virol. 86, 2057-2068.

Morin, M., Girard, C., Elazhary, Y., Fajardo, R., Drolet, R., Lagace, A., 1990. Severe proliferative and necrotizing pneumonia in pigs: A newly recognized disease. Can. Vet. J. 31, 837-839.

Morozov, I., Sirinarumitr, T., Sorden, S.D., Halbur, P.G., Morgan, M.K., Yoon, K.J., Paul, P.S., 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. 36, 2535-2541.

Nauwynck, H., Lefebvre, D., Misinzo, G., Meerts, P., Mateusen, B., Sanchez, R., Delputte, P., 2007. Pathogenesis of porcine circovirus 2 infections. In: Proceedings of the 38th Annual Meeting of the American Association of Swine Veterinarians, Orlando, Florida, USA, pp. 489-495.

Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. 81, 2281-2287.

Nielsen, J., Ladekjær Hansen, A.S., Bille-Hansen, V., Lohse, L., Bøtner, A., 2004. PCV2-associated disease following intrauterine infection. In: Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany, p. 14.

Nielsen, J., Vincent, I.E., Botner, A., Ladekaer-Mikkelsen, A.S., Allan, G., Summerfield, A., McCullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. 92, 97-111.

Nunez, A., McNeilly, F., Perea, A., Sanchez-Cordon, P.J., Huerta, B., Allan, G., Carrasco, L., 2003. Coinfection by Cryptosporidium parvum and porcine circovirus type 2 in weaned pigs. J. Vet Med. B Infect. Dis. Vet. Public Health 50, 255-258.

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.

Okuda, Y., Ono, M., Yazawa, S., Shibata, I., 2003. Experimental reproduction of postweaning multisystemic wasting syndrome in cesarean-derived, colostrum-deprived piglets inoculated with porcine circovirus type 2 (PCV2): investigation of quantitative PCV2 distribution and antibody responses. J. Vet. Diagn. Invest. 15, 107-114.

Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology 357, 175-185.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Rothschild, M., Kuhar, D., Lunney, J.K., Juhan, N.M., Meng, X.J., Halbur, P.G., 2009. Difference in severity of porcine circovirus type 2 (PCV2)-induced pathological lesions between Landrace and Pietrain pigs. J. Anim Sci.

Opriessnig, T., Ramamoorthy, S., Madson, D.M., Patterson, A.R., Pal, N., Carman, S., Meng, X.J., Halbur, P.G., 2008. Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. J. Gen. Virol. 89, 2482-2491.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Invest. 19, 591-615.

Opriessnig, T., Fenaux, M., Thomas, P., Hoogland, M.J., Rothschild, M.F., Meng, X.J., Halbur, P.G., 2006a. Evidence of breed-dependent differences in susceptibility to porcine circovirus type-2-associated disease and lesions. Vet. Pathol. 43, 281-293.

Opriessnig, T., Halbur, P.G., Yu, S., Thacker, E.L., Fenaux, M., Meng, X.J., 2006b. Effects of the timing of the administration of Mycoplasma hyopneumoniae bacterin on the development of lesions associated with porcine circovirus type 2. Vet. Rec. 158, 149-154.

Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. Vet. Pathol. 41, 624-640.

Opriessnig, T., Yu, S., Gallup, J.M., Evans, R.B., Fenaux, M., Pallares, F., Thacker, E.L., Brockus, C.W., Ackermann, M.R., Thomas, P., Meng, X.J., Halbur, P.G., 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet. Pathol. 40, 521-529.

Ostanello, F., Caprioli, A., Di, F.A., Battilani, M., Sala, G., Sarli, G., Mandrioli, L., McNeilly, F., Allan, G.M., Prosperi, S., 2005. Experimental infection of 3-week-old conventional colostrum-fed pigs with porcine circovirus type 2 and porcine parvovirus. Vet. Microbiol. 108, 179-186.

Park, J.S., Kim, J., Ha, Y., Jung, K., Choi, C., Lim, J.K., Kim, S.H., Chae, C., 2005. Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. J. Comp. Pathol. 132, 139-144.

Pensaert, M.B., Sanchez, R.E., Jr., Ladekjaer-Mikkelsen, A.S., Allan, G.M., Nauwynck, H.J., 2004. Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. Vet. Microbiol. 98, 175-183.

Pérez-Martín, E., Rovira, A., Calsamiglia, M., Mankertz, A., Rodriguez, F., Segalés, J., 2007. A new method to identify cell types that support porcine circovirus type 2 replication in formalin-fixed, paraffin-embedded swine tissues. J. Virol. Methods 146, 86-95.

Pesch, S., Schmidt, U., Ohlinger, V.F., 2000. Proliferative necrotizing pneumonia (PNP) is a result of coinfection with porcine reproductive and respiratory disease virus (PRRSV) and porcine circovirus type 2 (PCV2). In: Proceedings of the 16th International Pig Veterinary Society Congress, Melbourna, Australia, p. 581.

Phaneuf, L.R., Ceccarelli, A., Laing, J.R., Moloo, B., Turner, P.V., 2007. Porcine dermatitis and nephropathy syndrome associated with porcine circovirus 2 infection in a Yorkshire pig. J. Am. Assoc. Lab. Anim. Sci. 46, 68-72.

Pittman, J.S., 2008. Reproductive failure associated with porcine circovirus type 2 in gilts. J. Swine Health Prod. 16, 144-148.

Pogranichniy, R.M., Yoon, K.J., Harms, P.A., Sorden, S.D., Daniels, M., 2002. Case-control study on the association of porcine circovirus type 2 and other swine viral pathogens with postweaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. 14, 449-456.

Pogranichnyy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral. Immunol. 13, 143-153.

Quintana, J., Segalés, J., Rosell, C., Calsamiglia, M., Rodriguez-Arrioja, G.M., Chianini, F., Folch, J.M., Maldonado, J., Canal, M., Plana-Duran, J., Domingo, M., 2001. Clinical and pathological observations on pigs with postweaning multisystemic wasting syndrome. Vet. Rec. 149, 357-361.

Ramamoorthy, S., Meng, X., 2008. Porcine circoviruses: a minuscule yet mammoth paradox. Anim. Health Res. Rev. doi:10.1017/S1466252308001461.

Resendes, A.R., Majo, N., Segalés, J., Mateu, E., Calsamiglia, M., Domingo, M., 2004. Apoptosis in lymphoid organs of pigs naturally infected by porcine circovirus type 2. J. Gen. Virol. 85, 2837-2844.

Ritzmann, I., Majzoub, M., Hermanns, W., Heinritzi, K., Truyen, U., 2005. Clinical, haematological and clinical-chemical results in pigs with porcine dermatitis and nephropathy syndrome (PONS). Tieraerztl. Prax. Ausg. G Grosstiere Nutztiere 33, 299-303.

Rodriguez-Arrioja, G.M., Segalés, J., Calsamiglia, M., Resendes, A.R., Balasch, M., Plana-Duran, J., Casal, J., Domingo, M., 2002. Dynamics of porcine circovirus type 2 infection in a herd of pigs with postweaning multisystemic wasting syndrome. Am. J. Vet. Res. 63, 354-357.

Rose, N., Blanchard, P., Cariolet, R., Grasland, B., Amenna, N., Oger, A., Durand, B., Balasch, M., Jestin, A., Madec, F., 2007. Vaccination of porcine circovirus type 2 (PCV2)-infected sows against porcine Parvovirus (PPV) and

Erysipelas: effect on post-weaning multisystemic wasting syndrome (PMWS) and on PCV2 genome load in the offspring. J. Comp. Pathol. 136, 133-144.

Rosell, C., Segalés, J., Domingo, M., 2000a. Hepatitis and staging of hepatic damage in pigs naturally infected with porcine circovirus type 2. Vet. Pathol. 37, 687-692.

Rosell, C., Segalés, J., Ramos-Vara, J.A., Folch, J.M., Rodriguez-Arrioja, G.M., Duran, C.O., Balasch, M., Plana-Duran, J., Domingo, M., 2000b. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet. Rec. 146, 40-43.

Rovira, A., Balasch, M., Segalés, J., Garcia, L., Plana-Duran, 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.

Sanchez, R.E.Jr., 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. Diagn. Invest. 16, 175-185.

Sanchez, R.E.Jr., 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.

Sanchez, R.E.Jr., 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, 169-176.

Sarli, G., Mandrioli, L., Laurenti, M., Sidoli, L., Cerati, C., Rolla, G., Marcato, P.S., 2001. Immunohistochemical characterisation of the lymph node reaction in pig post-weaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. 83, 53-67.

Sarli, G., Mandrioli, L., Panarese, S., Brunetti, B., Segalés, J., Dominguez, J., Marcato, P.S., 2008. Characterization of interstitial nephritis in pigs with naturally occurring postweaning multisystemic wasting syndrome. Vet. Pathol. 45, 12-18.

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, 867-868.

Segalés, J., Allan, G.M., Domingo, M., 2006. Porcine Circovirus Diseases. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. Blackwell Publishing, Oxford, pp. 299-307.

Segalés, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. Anim. Health Res. Rev. 6, 119-142.

Segalés, J., Domingo, M., Chianini, F., Majo, N., Dominguez, J., Darwich, L., Mateu, E., 2004a. Immunosuppression in postweaning multisystemic wasting syndrome affected pigs. Vet. Microbiol. 98, 151-158.

Segalés, J., Rosell, C., Domingo, M., 2004b. Pathological findings associated with naturally acquired porcine circovirus type 2 associated disease. Vet. Microbiol. 98, 137-149.

Segalés, J., 2002. Update on postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome diagnostics. J. Swine Health Prod. 10, 277-281.

Segalés, J., Domingo, M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. Vet. Q. 24, 109-124.

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

Shibahara, T., Sato, K., Ishikawa, Y., Kadota, K., 2000. Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. J. Vet. Med. Sci. 62, 1125-1131.

Smith, W.J., Thomson, J.R., Done, S., 1993. Dermatitis/nephropathy syndrome of pigs. Vet. Rec. 132, 47.

Stanley, J., Bisaro, D.M., Briddon, R.W., Brown, J.K., Faquet, C.M., Harrison, B.D., Rybicki, E.P., Stenger, D.C., 2005. Geminiviridae. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 301-326.

Steiner, E., Balmelli, C., Herrmann, B., Summerfield, A., McCullough, K., 2008. Porcine circovirus type 2 displays pluripotency in cell targeting. Virology 378, 311-322.

Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2006. Demonstration of nicking/joining activity at the origin of DNA replication associated with the rep and rep' proteins of porcine circovirus type 1. J. Virol. 80, 6225-6234.

Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2001. Rep and Rep' protein of porcine circovirus type 1 bind to the origin of replication in vitro. Virology 291, 152-160.

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. Diagn. Invest. 13, 57-62.

Stockhofe-Zurwieden, N., Wichgers-schreurs, P., Hulst, M., Hansen, M.S., Van Der Meulen, J., Ruuls, L., Claassen, E., 2008. Early effects of PCV2 on porcine intestinal epithelial cells. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, p. 29.

Thacker, E.L., 2001. Immunology of the porcine respiratory disease complex. Vet. Clin. North Am. Food Anim. Pract. 17, 551-565.

Thibault, S., Drolet, R., Germain, M.C., D'Allaire, S., Larochelle, R., Magar, R., 1998. Cutaneous and systemic necrotizing vasculitis in swine. Vet. Pathol. 35, 108-116.

Thomson, G., Carman, S., 1991. Ontario. Proliferative and necrotizing pneumonia (PNP) of swine: the Ontario situation. Can. Vet. J. 32, 313.

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.

Tischer, I., Mields, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. 91, 271-276.

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., 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.

Todd, D., Bendinelli, M., Biagini, P., Hino, S., Mankertz, A., Mishiro, S., Niel, C., Okamoto, H., Raidal, S., Ritchie, B.W., Teo, G.C., 2005. Circoviridae. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 327-334.

Todd, D., 2000. Circoviruses: immunosuppressive threats to avian species: a review. Avian pathol. 29, 373-394.

Tomás, A., Fernandes, L.T., Valero, O., Segalés, J., 2008. A meta-analysis on experimental infections with porcine circovirus type 2 (PCV2). Vet. Microbiol. 132, 260-273.

Vetten, H.J., Chu, P.W.G., Dale, J.L., Harding, R., Hu, J., Katul, L., Kojima, M., Randles, J.W., Sano, Y., Thomas, J.E., 2005. Nanoviridae. In: Faquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsvier Academic Press, London, pp. 343-352.

Vincent, I.E., Carrasco, C.P., Guzylack-Piriou, L., Herrmann, B., McNeilly, F., Allan, G.M., Summerfield, A., McCullough, K.C., 2005. Subset-dependent modulation of dendritic cell activity by circovirus type 2. Immunology 115, 388-398.

Vincent, I.E., Carrasco, C.P., Herrmann, B., Meehan, B.M., Allan, G.M., Summerfield, A., McCullough, K.C., 2003. Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. J. Virol. 77, 13288-13300.

Wattrang, E., McNeilly, F., Allan, G.M., Greko, C., Fossum, C., Wallgren, P., 2002. Exudative epidermitis and porcine circovirus-2 infection in a Swedish SPF-herd. Vet. Microbiol. 86, 281-293.

Wellenberg, G.J., Stockhofe-Zurwieden, N., de Jong, M.F., Boersma, W.J., Elbers, A.R., 2004. Excessive porcine circovirus type 2 antibody titres may trigger the development of porcine dermatitis and nephropathy syndrome: a case-control study. Vet. Microbiol. 99, 203-214.

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. Diagn. Invest. 11, 530-532.

Yoon, K.J., Jepsen, R.J., Pogranichniy, R.M., Sorden, S., Stammer, R., Evans, L.E., 2004. A novel approach to intrauterine viral inoculation of swine using PCV type 2 as a model. Theriogenology 61, 1025-1037.

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.

Zhang, X., Ma, G.P., Li, Y.F., Jiang, X.T., He, J.L., Zhou, J.Y., 2009. Characterization of Monoclonal Antibody Against Replication-Associated Protein of Porcine Circovirus. DNA Cell Biol. 28, 23-29.