Technical University of Denmark

Association between PCV2 viral load in Serum Pools, Oral Fluid samples and Faecal Sock Samples in a Danish Finisher Herd

Master's Thesis
30 ECTS

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DTU Vet National Veterinary Institute

MSD Animal Health

SEGES

Preface

The following master's thesis marks the completion of the Veterinary master's degree for Katja Strøm Buse and Katrine Neumann. The master's thesis corresponds to 30 ECTS point.
The research study was made in cooperation with The National Veterinary Institute at the Danish Technical University (DTU-VET), under main supervision of Professor Lars Erik Larsen.
The master's thesis contains a literature study on PCV2 in general, but with focus on PCV2 viral load in serum, oral fluid and faecal sock samples. Furthermore, it contains a research study on associations between PCV2 load in serum, oral fluid and faecal sock samples and an assessment of the impact of individual pig´s viral load on a pooled serum sample.
The sampling for the research study was carried out in August 2017 and the master's thesis was completed in January 2018.

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Abstract

Porcine circovirus type 2 (PCV2) is often quantified by real-time polymerase chain reaction (qPCR) from pooled serum samples or penwise oral fluid (OF) samples. Since PCV2 can be detected in faeces, sock samples may be used as an alternative method for quantification of PCV2. The objectives of the study were to compare the PCV2 viral load in pooled serum samples, OF and faecal sock samples collected in the same pens, and to assess the impact of individual pig´s viral load on a pooled serum sample.

The study was carried out as a cross-sectional study in one Danish finisher herd, with a history of PCV2 infection, and a positive laboratory screening result for PCV2. Sock samples, OF samples and blood samples were collected for qPCR analysis from all individual pigs in each of 17 pens with pigs 14-15 weeks of age (Age-group 1) or 18-19 weeks of age (Age-group 2). Two serum pools from each pen were assembled in the laboratory. One pool including all the pigs in the pen (serum (all)), and one pool including only serum from pigs chewing the rope during OF collection (serum (chewers)). During collection of OF, 52.2% - 100% of the pigs in each pen chewed the rope. For Age-group 1, barely moderate correlations were observed between PCV2 viral load in OF and serum (all) (r =0.5, p=0.45) and OF and serum (chewers) (r = 0.51, p = 0.04). No correlations were observed for Age-group 2. Furthermore, the PCV2 viral load in OF was significantly higher than in serum pools, and a high variation in the PCV2 viral load in serum from individual pigs within pens was observed.

A barely moderate, negative correlation (r = -0.5, p = 0.04) between the PCV2 viral load in faeces from sock samples and serum (all), Age-group 2, while no significant correlation was observed for Age-group 1. Individual serum samples from seven pens revealed that four out of seven pens contained only one or two pigs with a PCV2 viral load as high as the matched serum (all).

The results from this study indicates, that neither a good agreement nor a strong correlation between the PCV2 results obtained from the different sample materials exists. The poor correlation may be because of differences in how the pools are made, and a result of a high variation in the PCV2 viral load in individual pigs within and between pens.

In addition, when interpreting serum pools, it is important to keep in mind that one pig with an apparently high PCV2 virus load can cause an increase in serum pool results.

Keywords: Porcine circovirus type 2, correlation, oral fluid, serum, faeces, diagnostics.

Resumé

Porcine circovirus type 2 (PCV2) kvantificeres ofte ved real-time polymerase chain reaction (qPCR) på poolede serum prøver eller ud fra stibaserede spytprøver opsamlet med reb. Eftersom PCV2 kan detekteres i fæces, kan sokkeprøver muligvis anvendes som en alternativ metode til kvantificering af PCV2. Formålet med dette studie var at sammenligne PCV2-niveauet i poolede serumprøver, spytprøver og fækale sokkeprøver, samt at vurdere hvilken påvirkning individuelle grises PCV2-niveau havde på den poolede serumprøves load.

Studiet blev udført som et tværsnitsstudie i en dansk slagtesvinebesætning, med en historik om PCV2 infektion samt et PCV2 positivt screeningssvar. I 17 stier blev der udtaget sokkeprøver, spytprøver og blodprøver fra alle grise i stien til qPCR-analyse, i hver af to aldersgrupper i alderen 14-15 uger (Aldersgruppe 1) og i alderen 18-19 uger (Aldersgruppe 2). Fra hver sti, blev der i laboratoriet udfærdiget to serum pools. Den ene pool bestod af serum fra alle grisene i stien (serum (all)) og den anden pool inkluderede kun serum fra grise, som tyggede i rebet under opsamlingen af spytprøven (serum (chewers)).

Under opsamlingen af spytprøven tyggede mellem 52.2% - 100% af grisene i rebet. I Aldersgruppe 1 blev svagt moderate korrelationer fundet mellem PCV2-niveauet i spyt og serum (all) ($r = 0,5$) og PCV2-niveauet i spyt og serum (chewers) ($r = 0,51$). Der blev ikke fundet nogen korrelation mellem PCV2-niveauet i serum og spyt i Aldersgruppe 2. Udover dette blev det også fundet at PCV2-niveauet i spyt var signifikant højere end i serum i begge aldersgrupper, og der blev observeret en høj variation i PCV2-niveauet i serum indenfor og i mellem stierne.

En svag moderat korrelation ($r = -0,5$) mellem PCV2-niveauet i sokkeprøver og serum (all) blev fundet i Aldersgruppe 2, imens ingen signifikant korrelation blev fundet for Aldersgruppe 1. Individuelle serum prøver fra syv stier afslørede, at der i fire ud af syv stier kun var én eller to grise med et PCV2-niveau lige så højt som niveauet i den matchede serum (all).

Resultaterne fra dette studie indikerer, at der hverken er en god overensstemmelse eller en stærk korrelation mellem PCV2-resultaterne i de forskellige prøvematerialer. De lave korrelationer, kan være som følge af forskelle i måden de forskellige pools fremkommer på, og den høje variation inden for og mellem stierne. Ydermere er det vigtigt at der ved fortolkning af resultat fra serum pools, huskes på, at én gris med et tilsyneladende højt PCV2-niveau, eventuelt kan forårsage en stigning i serum pool resultatet.

Nøgleord: Porcine circovirus type 2, diagnostik, spyt, serum, fæces, slagtesvin, korrelation.

Abbreviations

ADWG - Average Daily Weight Gain

CT value - Cycle Threshold value

DNA - Deoxyribonucleic acid

dNTPs - Deoxynucleotide Triphosphate

DTU-VET - Danish National Veterinary Institute at the Danish Technical University

ELISA - Enzyme-linked immunosorbent assay

IHC –Immunohistochemistry

ISH - In situ hybridization

mRNA – Messenger RiboNucleic Acid

NTC threshold – No template control

OF – Oral fluid sample

ORF - Open reading frame

PBS - Phosphate-buffered Saline

PCR - Polymerase chain reaction

PCV – Porcine Circovirus

PCVD – Porcine Circovirus associated Disease

PDNS – Porcine Dermatitis and Nephropathy Syndrome

PMWS – Postweaning Multisystemic Wasting Syndrome

PRDC - Porcine Respiratory Disease Complex

PRRS – Porcine Reproductive and Respiratory Syndrome

PRRSV - Porcine Reproductive and Respiratory Syndrome Virus

qPCR - Quantitative real-time PCR

RNA - RiboNucleic Acid

Serum (all) – serum pools containing serum from all the pigs in the pen

Serum (chewers) - serum pools containing serum from the pigs chewing the rope during collection of oral fluid.

SPF – Specific pathogen free

Contents

1 Introduction

In the late 1990s, the emergence of a new disease named postweaning mulitsystemic wasting syndrome (PMWS), lead to the discovery of PCV2 which differed in nucleotide and protein sequence from the formerly known PCV1 (Ellis *et al.*, 1998; Meehan *et al.*, 1998). PCV2 has since been associated with diseases such as PMWS, PDNS, reproductive failure and subclinical infection, collectively known as PCVD (Harms, Halbur and Sorden, 2002; Meng, 2013). Today the most common manifestation of PCV2 infection is subclinical PCVD (Segalés, 2012). Due to the potential negative impact on economically important production parameters, such as the average daily gain, the lean percentage and mortality rate (Young, Cunningham and Sanford, 2011; Alarcon, Rushton and Wieland, 2013), diagnosis of subclinical PCVD may be of interest. To support a suspected PCVD diagnosis, quantification of the PCV2 viral load in pooled serum samples is often used. The common practice in swine veterinarian practices is to investigate pools of serum from five pigs in the age groups to be investigated, thereby allowing for a herd diagnosis, but not an individual animal diagnosis (Nielsen, 2017).

In recent years, penwise oral fluid (OF) collection has become an attractive diagnostic method among swine veterinarians because it is a less labour-intensive method, are of lower stress to the animals than collection of serum, and provide the possibility to include a bigger proportion of animals in one sample, thereby further minimizing the cost associated with diagnostics (Prickett *et al.*, 2008, 2011; Ramirez *et al.*, 2012).

Since sock samples have shown to provide an easy and fast method for testing of some bacterial pathogens associated with intestinal infections (Pedersen *et al.*, 2015), and PCV2 virus is excreted in faeces (Shibata *et al.*, 2004; Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009), sock samples may provide an even easier method than collection of OF for determination of PCV2 viral load on a group level.

The following master's thesis contains a review on PCV2 in general, PCV2 viral load in serum, oral fluid and faecal sock samples and diagnostic possibilities. Furthermore, it contains a research study on associations between PCV2 viral load in serum, oral fluid and faeces from sock samples, and an assessment of the impact of individual pig´s viral load on a pooled serum sample.

2 Literature review

2.1 Historical background of PCV2

The first recognition of Porcine circovirus was in 1974 where a picorna-like virus contaminant of the porcine kidney cell line PK-15 (A TCC-CCL31) was described (Tischer *et al.*, 1974). Eight years later in 1982, the virus was characterized as a 17nm virus containing a covalently closed, circular, single-stranded DNA genome and a main capsid protein (Tischer *et al.*, 1982). Because antibodies against the virus were found only in pigs, it was suggested that the virus originated from pigs. Apparently, it was a virus not yet encountered. It was decided to name it Porcine circovirus (PCV), and it was assigned to the *Circoviridae* family (Tischer *et al.*, 1982; Allan and Ellis, 2000). Later, in a study by Tischer *et al.* (1986), it was discovered that antibodies against PCV were present in both slaughter pigs and 2-3 year old sows in Germany. Over the next 10 years, serum PCV antibodies were also demonstrated in pigs in Northern Ireland, Canada, New Zealand and Great Britain (Allan and Ellis, 2000). In experimental pathogenicity studies regarding PCV, neither clinical signs nor pathological lesions in organs from infected pigs were detectable. Due to lack of clinical disease and the widespread seropositivity in the herds, PCV was thought to be a ubiquitous non-pathogenic virus (Tischer *et al.*, 1986, 1995).

In the late 1990s, a new disease named PMWS emerged. The disease was first described in Western Canada, and later also in the United States and Europe (Chae, 2004; Segalés, 2012). In 1998 a circovirus-like virus was discovered in organs from pigs suffering from PMWS (Allan *et al.*, 1998; Ellis *et al.*, 1998). Meehan *et al.* (1998) discovered that the circovirus-like virus differed in nucleotide and protein sequence from the PCV PK-15 isolate discovered in 1974. This lead them to the conclusion that the circovirus associated with PMWS represented a new type of virus. The PCV PK-15 virus was named PCV1, and the PMWS associated virus was named PCV2 (Meehan *et al.*, 1998). The first cases of PMWS in Denmark appeared around 2001. Upon analysis of archived serum samples, it was found that PCV2 had been present in Denmark since 1982 (Dupont *et al.*, 2008).

2.2 Characteristics of Porcine Circovirus

Circoviruses are non-enveloped viruses with icosahedral symmetry containing a single-stranded circular DNA genome (Allan and Ellis, 2000). The genus *Circovirus* belongs to the family *Circoviridae* which, among others, contain PCV (Chae, 2004). Three types of PCV have been characterized including PCV1, PCV2 and the newly identified PCV3 (Tischer *et al.*, 1982; Meehan *et al.*, 1998; Palinski *et al.*, 2016). Despite the differences in pathogenicity between PCV1 and PCV2 they show an overall DNA sequence homology of 69% to 76%, with a homology between the viral

capsid proteins of 67% (Hamel *et al.*, 1998; Cheung, 2003; Chae, 2004). In comparison, PCV2 and PCV3 exhibit only 36 to 37% similarities between the capsid proteins (Palinski *et al.*, 2016).

PCV2 is 17nm in diameter with a genome size of about 1768 nucleotides (de Boisseson *et al.*, 2004). One capsid protein of 30 kDa, which is encoded by open reading frame 2 (ORF2), has been identified (Nawagitgul *et al.*, 2000). Together with open reading frame 1 (ORF1), which encodes for two proteins essential for DNA replication, these two constitute the main viral genes of PCV2 (Meehan *et al.*, 1998; Mankertz *et al.*, 2000; de Boisseson *et al.*, 2004). Furthermore, open reading frame 3 (ORF3) encodes a small protein, which is suggested to be involved in virulence modulation (Mankertz, 2012). The greatest variation in nucleotide sequence between different strains of PCV2 is found in ORF2 (Larochelle *et al.*, 2002).

PCV2 is a very stable virus exhibiting resistance to high temperatures and various pH conditions, but some disinfectants have proven effective in reducing PCV2 titters, including Virkon S (potassium peroxymonosulfate + sulfamic acid) and sodium hydroxide (Royer *et al.*, 2001; Patterson and Opriessnig, 2010).

Isolates of PCV2 can be divided into five subtypes named PCV2a, PCV2b, PCV2c, PCV2d and PCV2e (Xiao *et al.*, 2015; Davies *et al.*, 2016). Under experimental conditions it has been shown that cross-protection between PCV2a and PCV2b exists (Opriessnig *et al.*, 2008). PCV2a, PCV2b and PCV2c have been identified in Denmark. PCV2c was identified in archived serum samples from 1980, 1987 and 1990, and PCV2a in serum samples from 1993 and 1996 (Dupont *et al.*, 2008). Around 2001, when the first cases of PMWS were identified in Denmark, there was a shift in genotype occurrence from PCV2a to PCV2b (Vigre *et al.*, 2005; Dupont *et al.*, 2008). Analysis of PCV2 genome sequences from GenBank in 2007 showed that the same shift in genotype occurred worldwide around 2003, which have resulted in PCV2b being the dominant genotype worldwide (Dupont *et al.*, 2008; Xiao *et al.*, 2015).

2.3 Epidemiology and transmission

PCV2 is a ubiquitous virus in swineherds worldwide (Baekbo, Kristensen and Larsen, 2012). In 6234 serum samples from 185 grower/finisher sites in the United States a PCV2 prevalence of 82.6% were found. Out of the 185 swineherds 99.5% had at least one positive serum sample (Puvanendiran *et al.*, 2011). A similar prevalence of 100% was found in a study from United Kingdom, where all the 114 swineherds investigated were found positive for PCV2 antibodies or PCV2 DNA (Wieland *et al.,* 2010)

In naturally acquired infections, PCV2 can be transmitted either vertically or horizontally (Grau-Roma *et al.*, 2009; Dvorak *et al.*, 2013). The primary route of transmission is thought to be through oro-nasal contact (Meng, 2013), but PCV2 is also excreted in faeces (Patterson *et al.*, 2011), urine (Segalés *et al.*, 2005), semen (Larochelle *et al.*, 2000) and colostrum (Shen *et al.*, 2010).

Grau-Roma *et al.* (2009) suggested that the major spread of PCV2 under Danish field conditions occurs in pigs between 4 to 6 weeks of age. However, a relatively high percentage of piglets has been found positive for PCV2 virus in sera, nasal swabs and rectal swabs already in the first week of living, which indicates that transmission from sows to piglets also occurs (Grau-Roma *et al.*, 2009). The transmission from sows to piglets is thought to occur both horizontally and vertically (Grau-Roma *et al.*, 2009; Dvorak *et al.*, 2013).

Transmission of PCV2 within and between pens has been investigated in an experimental study by Andraud *et al.* (2008), who concluded that transmission was more effective within pens than between pens. This corresponds well to direct contact being the primary route of transmission (Dupont *et al.*, 2009).

PCV2 has been demonstrated in aerosols in swineherds (Verreault *et al.*, 2010), and transmission through air has been suggested by Dupont *et al.* (2009), since PCV2 infection in pigs located in an on-site control unit during a transmission study occured. However, the possibility and importance of transmission through air requires further investigation. In addition, although not considered the primary reason for introduction of PCV2 to a naïve herd, contaminated vehicles and vectors such as wild boars, mice and vaccines are thought to be possible routes of transmission (Rose *et al.*, 2012).

2.4 Pathogenesis

Even though PCV2 is associated with economically important diseases very little is known about the pathogenesis. As previously mentioned, the primary route of transmission in naturally infected pigs is thought to be through the oro-nasal route (Figure 1, a) (Dupont *et al.*, 2009; Meng, 2013). PCV2 has been detected in the nasal cavity, trachea-bronchial area, in the tonsils, in serum, in urine and in faeces (Shibata *et al.*, 2004; Segalés *et al.*, 2005). Furthermore, it has been detected in oral fluid (Prickett *et al.*, 2008, 2011) and in foetuses (Park *et al.*, 2005; Pittman, 2008). Lymphoid tissue is the main target of PCV2 and lymphoid lesions can be observed in pigs suffering from PMWS (Rosell *et al.*, 1999).

Yu *et al.* (2007) suggested that the initial replication of PCV2 occurs in the lymph nodes nearest the infection site, since capsid mRNA was detected earlier, and at a higher level in bronchial lymph nodes

compared to inguinal lymph nodes, in pigs experimentally infected through the nasal route (Figure 1, b).

Lymphocytes have been suggested to be the primary site of replication while the monocytes play a role in PCV2 persistence in the infected host (Yu *et al.*, 2007). PCV2 can also replicate in peripheral blood mononuclear cells, but there is some evidence that this only takes place transiently, since detection of capsid mRNA in these cells were only possible at one point during a study by Yu *et al.* (2007).

During infection, PCV2 spreads to various organs such as liver, lungs, spleen and thymus, and it is suggested to be either through haematogenous spread or by the means of peripheral blood mononuclear cells (Figure 1, c-d) (Rosell *et al.*, 1999; Yu *et al.*, 2007). The viremic period may persist for a long period. This was shown by Patterson *et al.* (2011) who were able to detect serum PCV2 DNA for 181 days in naturally infected pigs.

PCV2 infection in lymphoid tissue may result in lymphoid depletion and lymphopenia in the peripheral blood thereby causing immunosuppression (Meng, 2013). The lymphoid depletion is dependent on the amount of PCV2 antigen present in the lymphoid tissue, but it is still not known whether the depletion occurs as a consequence of virus-induced apoptosis, due to reduced proliferation in secondary lymphoid tissue or due to reduced production in the bone marrow (Opriessnig, Meng and Halbur, 2007; Meng, 2013).

The PCV2 associated disease which has gained the most attention is PMWS. The development of PMWS are not fully elucidated, but it has been proposed that neutralizing antibodies serve as protection for the development of PMWS (Brunborg *et al.*, 2010). In addition, co-infections have been proposed to increase the risk of developing PMWS (Pallarés *et al.*, 2002). Further studies on the importance of co-factors for the development of PMWS are required in order to fully understand the pathogenesis. For an overview of the proposed pathogenesis see Figure 1.

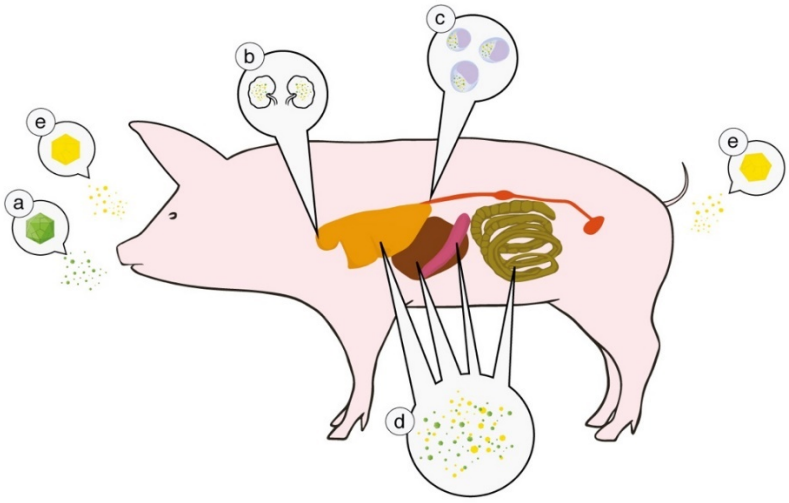

Figure 1: *An overview of the proposed pathogenesis: a) Oro-nasal transmission occurs (green virus particle). b) Infection is established in lymphoid tissue and replication of PCV2 primarily in the lymphocytes, within the lymph nodes occurs. c-d) Haematogenous spread to organs (lungs, liver, spleen, thymus). e) Virus shedding e.g. through saliva, faeces and urine (yellow particle) (Illustration by Rasmus Neumann).*

2.5 PCV2 associated diseases

Infection with PCV2 can give rise to different clinical presentations, collectively known as Systemic Porcine Circovirus Disease (PCVD). The diseases associated with PCV2 infection are PMWS, Porcine Dermatitis and Nephropathy Syndrome (PDNS), reproductive failure in sows and enteric disease. Furthermore, PCV2 is one of the primary agents contributing to the development of Porcine Respiratory Disease Complex (PRDC) (Opriessnig, Meng and Halbur, 2007).

2.5.1 PMWS

The most well-studied PCVD is PMWS. The clinical signs associated with PMWS are described as unthriftiness, pallor of the skin, icterus, dyspnoea and rough hair coat with an increase in post-weaning mortality rate especially due to weight loss and respiratory distress (Harding *et al.*, 1998). At necropsy, enlargement of at least one lymph node, non-collapsed lungs and pulmonary consolidation can be evident (Baekbo, Kristensen and Larsen, 2012).

The course of PMWS infection can be protracted starting with subtle clinical signs. The mortality rates can be as high as 30% in PMWS affected herds but are, among others, dependent on management on the farm and co-infections (Segalés, Allan and Domingo, 2005). The disease is most frequently observed in 2-4 month old pigs, but it has been reported in pigs ranging from 1- 6 months of age (Harding and Clark, 1997; Segalés, Allan and Domingo, 2005).

Because of the ubiquitous nature of PCV2 and the various differential diagnosis to wasting, presence of PCV2 and wasting in the pig is not enough to diagnose PMWS (Sorden, 2000; Patterson and

Opriessnig, 2010). The diagnosis is made based on: 1) the occurrence of specific clinical symptoms such as weight loss, wasting and respiratory distress, 2) the presence of PCV2-associated microscopic lesions such as presence of basophilic intracytoplasmic inclusion bodies in the lymph nodes, tonsils and Peyer patches of the ileum and moderate to severe lymphoid depletion, and 3) the presence of moderate to high amount of PCV2 antigen and nucleic acids in the microscopic lesions determined by immunohistochemistry (IHC) or in situ hybridization (ISH) (Harding and Clark, 1997; Sorden, 2000; Opriessnig, Meng and Halbur, 2007). For a definitive diagnosis, the findings described should be present in order to exclude differential diagnosis, such as PRRS, *M. hyopneumoniae* and post weaning diarrhoea as a cause of the clinical symptoms present (Harding and Clark, 1997). However, this method is quite labour intensive, and quantification of PCV2 by qPCR in serum for confirming or discarding a PMWS case on an individual animal level has therefore been suggested. The most supported cut-off value for discriminating healthy pigs and PMWS affected pigs are 7 $\log_{10}$ PCV2 copies/ml serum (Brunborg, Moldal and Jonassen, 2004; Olvera *et al.*, 2004; Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009). However, quantification of PCV2 viral load in serum cannot substitute histopathology and detection of PCV2 antigen in tissues for individual PMWS diagnosis, because of low diagnostic sensitivities and specificities (Grau-Roma *et al.*, 2009).

2.5.2 PDNS

PDNS typically affects nursery and finisher pigs, but can affect adult pigs as well. The clinical signs of PDNS are anorexia, depression, ventrocaudal subcutaneous oedema and high mortality rates among affected pigs (Rosell *et al.*, 2000). The most prominent clinical sign in the acute phase of PDNS are the red-to-purple papules on the skin often located on the hind limbs, on the abdomen and in the perineal area (Wellenberg *et al.*, 2004; Segalés, Allan and Domingo, 2005). At necropsy, the kidneys appear enlarged, grey-brown in colour and with petechial or multifocal white spots. At histopathology, acute and chronic exudative glomerulonephritis accompanied by interstitial nephritis are often present (Wellenberg *et al.*, 2004). Even though PDNS has been well described, there is still a need for more research concerning the role of PCV2 in causing the disease, as a recent study has suggested that PCV3 might contribute to the development of the disease (Palinski *et al.*, 2016).

2.5.3 PRDC

PCV2 has been identified as a contributor to the development of Porcine respiratory disease complex (PRDC), together with other viral pathogens such as Porcine reproductive and respiratory syndrome Virus (PRRSV) and swine influenza virus (SIV) (Harms, Halbur and Sorden, 2002). Coughing,

dyspnoea and lethargy are often prominent signs of the disease complex (Harms, Halbur and Sorden, 2002).

2.5.4 PCV2 associated reproductive disorder

PCV2 associated reproductive failure is characterized by abortions and stillbirth, where PCV2 can be identified in lymph nodes and the heart of aborted and stillborn foetuses. In some cases, necrotizing myocarditis can be seen in the aborted foetuses (West *et al.*, 1999; Park *et al.*, 2005; Pittman, 2008)

2.5.5 PCV2 associated enteric disease

Enteric disease, with diarrhoea as the main clinical symptom, has also been associated with PCV2 infection (Jensen *et al.*, 2006; Segalés, 2012; Baró, Segalés and Martínez, 2015). Colitis is the primary cause of diarrhoea, but enterocolitis and enteritis have also been observed in PCV2 associated enteric disease (Baró, Segalés and Martínez, 2015).

2.5.6 PCV2 subclinical infection

Even though PCV2 can give rise to the above mentioned diseases, the majority of the infected herds are subclinical infected, and therefore no PCV2 associated clinical signs are present (Segalés, 2012). However, subclinical PCV2 infections may be economically important as production parameters such as the average daily gain, the lean percentage and mortality rate can be negatively affected (Young, Cunningham and Sanford, 2011; Alarcon, Rushton and Wieland, 2013). The importance of the subclinical infection on the average daily weight gain (ADWG) has shown to be dependent on the PCV2 viral load, as López-Soria *et al.* (2014) found that, at a higher PCV2 virus load the ADWG was lower. Vaccination against PCV2 has shown to improve the aforementioned production parameters in growing pigs (Young, Cunningham and Sanford, 2011).

2.6 PCV2 and co-infections

PCV2 has been demonstrated as the causative agent of PCVD in experimentally infected pigs and piglets (Bolin *et al.*, 2001; Ladekjær-Mikkelsen *et al.*, 2002; Hasslung *et al.*, 2005). Although it has been established that PCV2 is essential in the development of PMWS, it is likely that cofactors are necessary for the expression of the disease, since experimental infection with PCV2 alone has proven to present a challenge in experimental reproduction of PMWS (Opriessnig, Meng and Halbur, 2007; Meng, 2013). It has been done most consistently when PCV2 was inoculated together with other swine pathogens such as Porcine parvovirus, porcine respiratory and reproductive virus and *Mycoplasma hyopneumonia* (Allan *et al.*, 1999; Rovira *et al.*, 2002; Opriessnig *et al.*, 2004). A field

study, carried out in the United States between 2000 and 2001, support the hypothesis that coinfections with other swine pathogens are necessary for development of severe PMWS. The study showed that out of 484 diagnosed cases of PMWS only 1.9% were cases of singular PCV2 infection. The most common findings were PRRS and PCV2 coinfection (33.3%), PCV2 and *M. hyopneumoniae* coinfection (19%) and PCV2, PRRS and *M. hyopneumoniae* coinfections (15.9%) (Pallarés *et al.*, 2002).

Co-infections may be an explanation for the development of clinical illness, but other factors such as a high viral load seen in PMWS cases or environmental factors may also be of importance in exacerbation of the illness (Brunborg, Moldal and Jonassen, 2004; Segalés, Allan and Domingo, 2005).

2.7 Strategies to control PCV2 infection

Various strategies to control PCV2 infection have been identified, including vaccinations. Several vaccines have been developed in order to reduce the economic losses associated with PCV2 infection. At present, there are four commercial vaccines available on the Danish market, all of which can be administered at 3 weeks of age (Medicintildyr.dk, 2017). A meta-analysis of studies regarding the effects of PCV2 vaccines have shown that all four vaccines have proven to be effective in increasing the average daily gain, and reducing mortality rate in grower/finisher pigs (Kristensen, Baadsgaard and Toft, 2011). Vaccination has not proved to be effective in reducing mortality rate in nursery pigs alone, but it can be reduced quite effectively in the nursery-finishing and finishing phase (4-5%-point). From an economic perspective, this reduction might be the main reason for vaccination as the increase in the average daily gain, although statistically significant, was found to be quite low (Kristensen, Baadsgaard and Toft, 2011). Alarcon *et al.* (2013) have reported the economic efficiency of different control strategies. It was found that in moderately PMWS affected farms and subclinically infected farms, vaccination was the most cost-efficient strategy, whereas a combination of biosecurity improvements and vaccination was the most cost-effective control strategy in severely PMWS affected herds (Alarcon *et al.,* 2013).

2.8 Sample materials for PCV2 detection and quantification

PCV2 can be detected and quantified by qPCR in different sample materials, including serum, OF and faeces. The previously mentioned methods used to ensure a correct diagnosis of PMWS on an individual animal level (see section 2.5.1) are quite labour intensive and does not allow for diagnosis on live animals, as it involves necropsies and histological examinations on tissue from individual pigs (Harding and Clark, 1997). Because of this, it has been investigated whether qPCR on serum could

be used as an alternative method for diagnosing PMWS (Brunborg, Moldal and Jonassen, 2004; Olvera *et al.*, 2004; Segalés *et al.*, 2005; Dupont *et al.*, 2009). PCV2 infections in the herds are usually subclinical and, as previously mentioned, may adversely affect the mortality rate and average daily gain, thereby making subclinical PCVD economically important (Alarcon, Rushton and Wieland, 2013). For diagnosis of suspected subclinical PCVD, quantification of the PCV2 viral load in pooled serum samples instead of individual serum samples are used, thereby minimizing the costs associated with diagnostics (Nielsen, 2017). In swine veterinary practice the most common method is to pool serum from five individual pigs. The diagnosis of subclinical PCVD is then made on a herd level instead of on an individual animal level (Nielsen, 2017).

Another method for obtaining a sample, where a higher proportion of pigs are represented, is using a cotton rope to collect an OF sample (see Figure 2). OF has gained some attention, because it is a less labour-intensive method, are of lower stress to the animals than collection of serum and provide the possibility to include a bigger proportion of animals in one sample, thereby further minimizing the cost associated with diagnostics (Hernandez-Garcia *et al.*, 2017). The method for obtaining an OF sample comprises of hanging a cotton rope in the pen. This allows individual pigs to chew on the rope and thus contribute to the overall sample. The rope hangs for 30 minutes, after which it is wrung to release the OF from the rope. The PCV2 viral load can then be quantified by qPCR analysis (Prickett *et al.*, 2008).

Figure 2: *Collection of oral fluid by a cotton rope. The Individual pigs are allowed to chew the rope for 30 minutes before it is wrung to release OF (Picture: http://landbrugsavisen.dk/svin/dit-foder-kan-være-skyld-i-falske-laboratoriesvar)*

As in the case of OF samples, sock samples also give the possibility to include a larger number of pigs in one sample. Sock samples are collected by walking in the faecal material in the pen, while wearing an absorbing felt sock on the boot (Figure 3)(Pedersen *et al.*, 2015). Since sock samples have shown to be an easy and fast method for testing for some intestinal pathogens (Pedersen *et al.*, 2015) and PCV2 virus is excreted in faeces (Shibata *et al.*, 2004; Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009), sock samples may be an even easier method than collection of OF, for determination of PCV2 viral load on a group level.

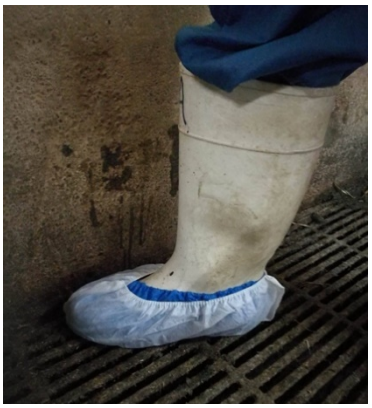

Figure 3: *Sock samples are collected by walking in the faecal material in the pen, while wearing an absorbing felt sock, outside of a plastic sock, on the boot. (Picture by Maja Kobberø)*

In the following sections an overview of the PCV2 antibody and viral load in serum, OF and faeces and the use in relation to diagnostics will be reviewed.

2.8.1 Serum

2.8.1.1 PCV2 antibodies and PCV2 virus in serum

PCV2 has been detected in natural infected pigs for up to 181 days (Patterson *et al.*, 2011). After infection, an increase in the PCV2 viral load in serum occurs, after which a slow but gradual decline can be observed (Brunborg *et al.*, 2010; Patterson *et al.*, 2011). The PCV2 viral load in serum has been shown to be significantly higher in PMWS affected pigs and in pigs prior to PMWS outbreak compared to healthy pigs (Grau-Roma *et al.*, 2009). Likewise, it has been shown that the PCV2 viral load in subclinically infected pigs is higher than in healthy pigs (Segalés *et al.*, 2005). A high PCV2 viral load may be present in pigs from non-PMWS herds. In a herd free from PMWS, Brunborg *et al.* (2010) found an average PCV2 viral load of 10^6 PCV2 copies/ml serum, with individual pigs exceeding the proposed cut-off value of 10^7 PCV2 copies/ml serum. A similar average PCV2 viral load was found in a PMWS positive herd. However, the pigs that developed PMWS had in general a lower antibody response, which declined until the onset of PMWS (Brunborg *et al.*, 2010).

PCV2 specific antibodies can be present as early as in the first week of a piglets life, due to maternal antibodies (Sibila *et al.*, 2004; Grau-Roma *et al.*, 2009). Grau-Roma *et al.* (2009) have shown that the PCV2 antibody level declines from the first week of life until around week 6-7 of life while a concurrent increase in the PCV2 viral load in serum can be observed. There are some evidence that antibody levels are higher or can be detected in greater percentages in healthy pigs compared to pigs suffering from PMWS (Sibila *et al.*, 2004; Grau-Roma *et al.*, 2009; Brunborg *et al.*, 2010). Despite of an active immune response against PCV2, viremia may still be present (Grau-Roma *et al.*, 2009; Brunborg *et al.*, 2010).

2.8.1.2 Serum pools for diagnosis of PCVD

As previously mentioned, the most common manifestation of PCV2 infection is subclinical PCVD (Segalés, 2012), and because of the potential economic impact on production parameters, quantification of the PCV2 viral load may be of relevance when interventions such as vaccinations are considered. Because the detection of PCV2 specific antibodies not in itself is indicative of an existing infection, the detection of antibodies is therefore often not relevant when the aim is to evaluate active infection (Allan and Ellis, 2000). qPCR on pooled serum samples are often used for quantification of PCV2 viral load on a herd level. The advantage of pooled serum samples is that it

allows for a larger number of pigs to be represented for the same laboratory costs, making it more economically attractive for the farmer (Cortey *et al.*, 2011). Pooling of serum consists of taking the same amount of serum from each individual sample and pool into one sample. The pooling can take place in the herd, or at a laboratory (Nielsen, 2017).

The use of serum pools does not allow for determination of PCV2 viral load on an individual animal level, which is illustrated by the fact that a positive serum pool may contain PCV2 negative pigs (Nielsen, 2017). This has been shown by Nielsen (2017) who found that a positive theoretical pool of five pigs often contained PCV2 negative pigs. On the other hand, a negative pool did not contain PCV2 positive pigs (Nielsen, 2017).

qPCR on serum pools may be used for identification of highly viremic pigs, and a cut-off value of 6.7 $\log_{10}$ PCV2 copies/ml serum in a pool of five pigs, for identification of one or more pigs with a viral load > 7 $\log_{10}$ PCV2 copies/ml serum, has been proposed (Nielsen, 2017).

2.8.2 Oral fluid

Another method for obtaining a sample in which a larger proportion of animals are represented, is by collection of OF as described by Prickett *et al.* (2008). This method allows for a whole pen to be represented in one sample. Because it is possible and valid to detect and quantify the amount of different viral pathogens such as PRRSv and PCV2 in OF, it allows for a time and cost effective method to monitor both virus and antibodies in the herd (Prickett *et al.*, 2008, 2011; Ramirez *et al.*, 2012).

2.8.2.1 PCV2 viral load and antibodies in oral fluid and oral/nasal secretions

In addition to containing secretion from the oral cavity, OF may also contain secretion from the respiratory system including the nasal cavity (Hernandez-Garcia *et al.*, 2017). PCV2 can be detected in the nasal and oral cavity (Sibila *et al.*, 2004; Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009), and detection of PCV2 in the nasal cavity prior to PCV2 viremia has been shown (Sibila *et al.*, 2004). Sibila *et al.* (2004) suggested that the earlier detection of PCV2 in nasal secretions compared to detection in serum most likely was due to early infection, since 80% of the pigs positive for PCV2 virus in nasal swabs, but not in serum, were under 3 months of age.

The PCV2 viral load in nasal and tonsillar swabs has been shown to be higher in PMWS and subclinically infected pigs compared to healthy pigs (Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009).

Furthermore, in four naturally infected pigs kept under experimental conditions, a higher PCV2 viral load in oral swabs compared to nasal swabs and serum was found from day 28 through day 84 of the study period (Patterson *et al.*, 2011). PCV2 was consistently detected in both serum, nasal and oral

swabs until day 126, after which PCV2 was detected intermittently in the three samples (Patterson *et al.*, 2011).

Like for the PCV2 viral load in oral swabs, a higher PCV2 viral load in OF samples compared to serum has also been shown (Oliver-Ferrando *et al.*, 2016; Nielsen, 2017). Furthermore, an earlier detection of PCV2 in OF samples compared to serum samples has also been shown (Kim, 2010). Nevertheless, OF has proved to be a good method for herd monitoring of PCV2 virus and PCV2 antibodies (Prickett *et al.*, 2011). Prickett *et al.* (2011) investigated both PCV2 viral load and PCV2 antibody occurrence in OF from experimentally infected pigs. The OF samples were positive for PCV2 on day two after intramuscular and nasal inoculation and remained positive during the study period of 98 days. Seroconversion occurred between day 14 and 21 after inoculation, after which PCV2 antibodies were detectable through the rest of the study period. This means that in addition to prolonged shedding of PCV2 in OF, the study also indicated that PCV2 infection may persist even in the presence of an active immune response (Prickett *et al.*, 2011).

Few studies have suggested viral load cut-off values for discriminating between PMWS affected pigs and healthy pigs by nasal swabs. Viral load cut-off values of 9.2 $\log_{10}$ PCV2 copies/ml sample (Danish study) and of 5.9 $\log_{10}$ PCV2 copies/ml sample (Spanish study) have been suggested by Grau-Roma *et al.* (2009). The differences in cut-off values between the Spanish and Danish investigations are highly indicative of the dependence on collection procedures and methods for the viral load detected (Grau-Roma *et al.*, 2009). Nevertheless, from the Danish study it seems that the PCV2 viral load in nasal swabs needs to be higher compared to serum when diagnosing PMWS.

2.8.2.2 Use of oral fluid for quantification of PCV2 load on a herd level

When collecting OF, there may be differences in the number of pigs chewing the rope, and thereby the number of pigs being represented in the sample (Seddon, Guy and Edwards, 2012). Various factors may influence the likelihood of pigs being represented in the sample, including environmental enrichment (Scott *et al.*, 2006), housing systems (Seddon, Guy and Edwards, 2012) and age of the pigs (Hernandez-Garcia *et al.*, 2017). Nevertheless, Seddon, Guy and Edwards (2012) showed that in grower pigs, 80% of pigs in a pen had chewed the rope after 60 minutes, with the greatest increase of pigs chewing during the first 30 minutes.

qPCR analysis on OF samples is a sensitive and specific method for monitoring PCV2 at pen level, which was shown by Prickett *et al.* (2011), who reported a sensitivity of 98% and a specificity of 100%. These sensitivity and specificity estimations were based on OF samples collected from 57 pens housing PCV2 inoculated pigs and 19 pens housing non-inoculated pigs (Prickett *et al.*, 2011). Despite the high sensitivity/specificity reported by Prickett *et al.* (2011) a risk of both false negatives

and an underestimation of the PCV2 viral load exists, as OF may contain factors that can have an inhibitory effect on PCR (Ochert *et al.*, 1994). However, this can often be handled by diluting the sample before purification (Kubista *et al.*, 2006).

Few previous studies have investigated how the PCV2 viral load in OF correlates with the PCV2 viral load in serum, and these studies have obtained somewhat different results. Oliver-Ferrando *et al.* (2016) did not find a significant correlation between PCV2 viral load in serum and OF, when investigating serum samples from two to four pigs and OF samples from 11 to 23 pigs (see Table 1). However, the correlation was based only on PCV2 qPCR positive samples from groups of vaccinated and unvaccinated nursery and fattening pigs, and it was not taken into account that samples could not be considered independent. In a cross-sectional study with follow-up, where four consecutive samples were collected in the same pens, a low, but significant correlation was obtained in the 1st sampling, but no correlation was obtained in the 2nd, 3rd and 4th sampling (see Table 1) (Nielsen, 2017). Higher correlations between PCV2 viral load in OF and serum have also been obtained (see Table 1) (Kim, 2010; Nielsen, 2017). However, both correlations were calculated from samples collected from pens across different age groups. Nevertheless, the differences in the correlation coefficients obtained, has been suggested to be because of differences in the proportion of pigs contributing to OF samples compared to the pooled serum sample (Nielsen, 2017). This could be a reasonable explanation since a good correlation between PCV2 viral load in nasal cavity and PCV2 viral load in serum has been obtained on an individual animal level (see Table 1).

A determination of the correlation between PCV2 viral load in OF and in a pooled serum sample, if exactly the same pigs are sampled by both methods, is, to the authors´ knowledge, currently lacking.

Table 1: *Overview of the correlation coefficients between PCV2 viral load in nasal swabs and serum (individual animal level) and OF and serum (pooled samples) obtained in previous studies.*

Sample	Method	Correlation coefficient	Reference
Nasal swabs and serum	Individual animal level	r = 0.693 (Spanish), r = 0.663 (Danish)	Grau-Roma *et al.* (2009)
OF and serum	4/5 pig in serum pool, 20-30 pigs in OF sample	ρ = 0.69 (Herd 1), ρ = 0.39 (Herd 2, 1st sampling),	(Nielsen, 2017)
OF and serum	2-4 pigs in serum, 11-23 pigs in OF sample	r = 0.76 (p= 0.13)	(Oliver-Ferrando *et al.*, 2016)
OF and serum	5 pigs in serum pool, 20-30 pigs in OF	r = 0.78	(Kim, 2010)

2.8.3 Faeces

2.8.3.1 Shedding of PCV2 in faeces

As previously mentioned, PCV2 is excreted in faeces and can be detected and quantified by PCR and qPCR analysis on faecal material (Shibata *et al.*, 2004; Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009). PCV2 viral load in faecal swabs have been shown to be higher in PMWS affected pigs than in healthy pigs, but not higher in subclinically infected pigs (Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009).
In comparison to excretion through the nasal route, the prevalence of pigs excreting PCV2 in faeces and the load of PCV2 in faeces are found to be lower, although both oral/nasal and rectal swabs are suitable for monitoring the excretion (Segalés *et al.*, 2005; Grau-Roma *et al.*, 2009). In pigs viremic for a longer period of time, shedding in faeces have shown to occur intermittently (Grau-Roma *et al.*, 2009).

2.8.3.2 Comparison of PCV2 viral load in rectal and faecal swabs and PCV2 viral load in serum

Most studies involving PCV2 virus in faeces have investigated excretion patterns on an individual animal level using rectal or faecal swabs. Significant correlations between PCV2 viral load in faecal swabs and serum has been found by Grau-Roma *et al.* (2009) (see Table 2). It has also been reported by Segalés *et al.* (2005), that in pigs with severe PCV2 associated lesions in organ materials, a higher PCV2 viral load in both serum and in rectal swabs were present.
Few studies have suggested a PCV2 viral load cut-off value for discriminating between healthy pigs and PMWS affected pigs at an individual animal level by the means of rectal swabs. Segalés *et al.* (2005) suggested a viral load cut-off of 5 $\log_{10}$ PCV2 copies/ng. A similar cut-off value of 5.9 $\log_{10}$ PCV2 copies/ml sample suggested by Grau-Roma *et al.* (2009) in a Spanish study. The cut-off value may, however, be highly dependent on sampling procedure and methods used, since the proposed cut-off value was estimated to 8.1 $\log_{10}$ PCV2 copies/ml sample in a Danish study (Grau-Roma *et al.*, 2009).

Table 2: *Correlations between PCV2 viral load in faecal swabs and serum obtained in previous studies.*

Sample	Method	Correlation coefficient	Article
Faecal swab and serum	Individual animal level	r = 0.608 (Spanish), r = 0.736 (Danish)	Grau-Roma *et al.* (2009)

2.8.3.3 The use of sock sampling for diagnostic purposes

Another method for collection of faecal material is sock sampling. The method is described in section 2.8. Sock sampling is used in the swine production as well as in the poultry production to test for different bacterial pathogens, such as *Lawsonia intracellularis*, *Brachyspira piloscicoli* and *Escherichia coli* F4 and F18 in pigs (Pedersen *et al.*, 2015) and *Campylobacter* spp. and *Salmonella* spp. in broilers (Skov *et al.*, 1999; Matt *et al.*, 2016). The utility and performance of sock samples in pigs have been investigated by Pedersen *et al.* (2015), who concluded that sock samples are an easy and fast method for testing for *L. intracellularis*, *B. piloscicoli* and *E. coli* F4 and F18, with an acceptable performance and repeatability (Pedersen *et al.*, 2015). If the same applies for sock samples when used to quantify the PCV2 viral load, it may be an easier alternative to OF and serum collection. Because the determination of PCV2 viral load in individual faecal samples is valid, it is reasonable to assume that faeces collected by sock samples also will provide a valid method PCV2 determination. To the authors knowledge, quantification of PCV2 viral load by qPCR on faeces from sock samples, and determination of the correlation between PCV2 in sock samples and PCV2 in serum from the same pen, has not been investigated.

2.9 Diagnostic methods and procedures

Depending on the material to be examined, there are different diagnostic methods for the detection and quantification of PCV2 virus or PCV2 antibodies. Over time various methods have been developed, of which PCR is one of the most important molecular techniques used today (Quinn *et al.*, 2011). When the aim is to evaluate active infection, methods for antigen or DNA sequence detection is often used, as antibodies in itself are not indicative of existing infection (Allan and Ellis, 2000). Therefore, diagnostic methods used for antibody detection are especially useful for epidemiological surveys and retrospective diagnosis of viral infections (Quinn *et al.*, 2011).

This section describes the basic principles of different diagnostic methods for antigen, antibody and nucleic acid sequence detection and quantification.

2.9.1 In situ hybridization and immunohistochemistry

ISH is one of the methods used to confirm a suspected PMWS diagnosis (Sorden, 2000). The technique is especially useful for the detection of PCV2, as the virus is difficult to cultivate and are non-cytopathic in cell-cultures (McNeilly *et al.*, 1999). The method is used to evaluate gene expression and detect nucleic acids within tissues such as lymph nodes, liver or spleen (Sorden, 2000; Jensen, 2014). The essence of the technique is that a labelled nucleic acid probe hybridizes with a complementary sequence of DNA or RNA within the tissue sample (Wilcox, 1993). Before the

hybridization procedure, the tissue is cut into thin slices and placed on a special glass slide (Wilcox, 1993). The tissue needs to be fixated, to ensure the preservation of tissue morphology, and treated with various chemicals, for example proteases which increases target accessibility (Jensen, 2014). Paraffin-embedded, formalin fixed tissue sections can be used for investigations (Ramos-Vara and Miller, 2014). After hybridization the glass slide can be examined by autoradiography or by different microscopic methods (Jensen, 2014).

IHC can also be used to confirm a suspected PMWS diagnosis (Sorden, 2000). The same basic principles used in ISH applies to IHC, but the main difference between the two methods is that IHC is used to detect antigens by the means of immunoglobulins which binds to the antigens (Ramos-Vara and Miller, 2014). The antibody-antigen complexes elicit a light that can be investigated through the use of light or fluorescent microscopy (Ramos-Vara and Miller, 2014).

The use of the two methods for confirming a suspected PMWS diagnosis has been investigated by Sorden *et al.* (1999). When comparing the methods, IHC proved to be a faster and less labour intensive technique. However, both can be used as the sensitivity are equally high for both methods (Sorden *et al.*, 1999).

2.9.2 Enzyme-linked immunosorbent assay

Several ELISA methods have been developed for detection and quantification of antigens or antibodies in biological fluids. In general, the methods are based on antibody-antigen reactions, which are made visible through a colour change obtained from enzyme-linked conjugate and enzyme substrates (Aydin, 2015).

The ELISA method is a sensitive and specific method (Walker *et al.*, 2000), and it has been used in several epidemiological studies regarding PCV2 (Sibila *et al.*, 2004; Grau-Roma *et al.*, 2009; Prickett *et al.*, 2011; Ramirez *et al.*, 2012). At the Danish National Veterinary Institute at Danish Technical University of Denmark (DTU-VET), the method which is currently used for detection of PCV2 specific antibodies is the SerELISA® PCV2 Ab Mono Blocking (Synbiotics Europe, 2017).

2.9.3 Polymerase Chain Reaction

PCR is one of the most important molecular techniques in modern diagnostics. It is an exponential process which allows for amplification of specific DNA fragments in a simple enzymatic reaction, generating a large amount of identical copies (Arnheim and Erlich, 1992). PCR is a sensitive method as it only requires a small amount of DNA in a sample to generate enough copies for further analysis (Garibyan and Nidhi, 2013). qPCR allows for quantification of the PCR product during the course of the reaction by the measurement of the fluorescent signal (Kubista *et al.*, 2006). Before PCR analysis

of biological samples, purification and dilution are essential to minimize the risk of inhibition (Kubista *et al.*, 2006).

Several PCR and qPCR assays have been developed for viral and bacterial agents, including PCV2 (Quinn *et al.*, 2011).

2.9.3.1 Conventional Polymerase Chain Reaction

For each PCR assay two target sequence specific oligonucleotide primers, dNTPs, DNA polymerase, a DNA template and a buffer containing magnesium ions are required (Kubista *et al.*, 2006). In general, the reaction is carried out in a series of cycles, all of which consists of three repeated phases (see Figure 4) (Kubista *et al.*, 2006). The first phase occurs at 95 °C, where the double-stranded DNA denatures. In the second phase, the oligonucleotide primers anneal to the complementary target DNA sequence of the template typically at 50-60 °C, but the actual temperature depends on the primer sequence (Kubista *et al.*, 2006). The DNA polymerase elongates the primers by adding nucleotides to the growing DNA strand, thereby producing full amplicons (Arnheim and Erlich, 1992). The temperature during this phase is typically 72 °C, depending on the polymerase used. During the cycling the number of amplicons increases exponentially, reaching a plateau after varying number of cycles (Arnheim and Erlich, 1992). For the analysis and visualization of the PCR product, agarose gel electrophoresis is the easiest and the most widely used method, which allows determination of the size of the PCR product (Garibyan and Nidhi, 2013).

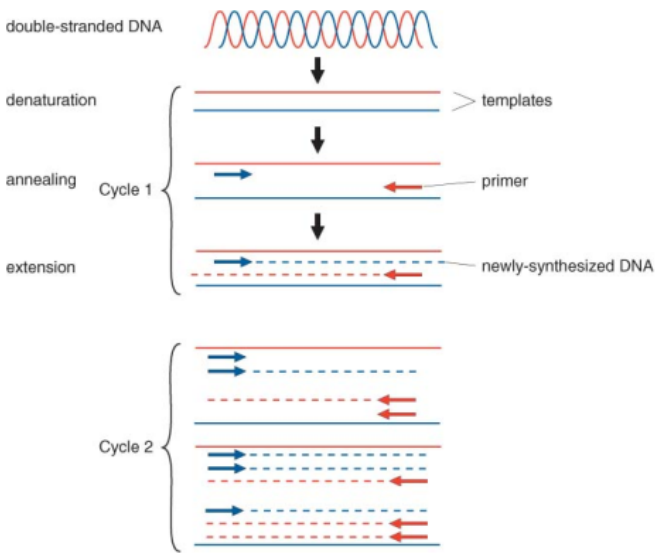

Figure 4: *The PCR reaction. The first two cycles are showed for illustration. In each cycle, the first phase consists of denaturing of the double- stranded DNA (denaturation). In the second phase the oligonucleotide primers anneal to the complementary target DNA sequence of the template (annealing). In the third phase, the DNA polymerase elongates the primers by adding nucleotides to the growing DNA strand, thereby producing full* amplicons (extension)(Quinn *et al.*, 2011, p. 288).

2.9.3.2 Quantitative real time PCR

A newer variant of PCR, is the qPCR technique, whereby the amount of PCR product is monitored using a fluorescent signal while the product is being synthesized i.e. in real time. This allows for the calculation of the number of DNA molecules initially present in the sample (Kubista et al., 2006).
To detect the fluorescent signal monitored during the reaction, several types of chemistries have been developed.

Oligonucleotide probes with attached fluorophores are widely used as a fluorescent source as it increases the specificity of the qPCR (Klein, 2002). One example is the *TaqMan* hydrolysis probe (also called a dual labelled probe). The probe has a reporter fluorophore and a quencher fluorophore attached at each end of the probe. The signal from the reporter fluorophore is absorbed by the quencher due to the close proximity of the two (Valasek and Repa, 2005). During elongation, the probe is degraded by the *Taq* DNA polymerase, which causes the reporter and quencher to be separated and thereby the reporters energy and fluorescent signal are released. Thus, an increase in the fluorescent signal will occur (Valasek and Repa, 2005).

The qPCR chemistry used at DTU-VET for quantification of PCV2 is the *Primer-Probe Energy Transfer* (*PriProET)*. This assay is based on fluorescence resonance energy transfer (FRET) from a donor to a reporter fluorophore (Hakhverdyan *et al.*, 2006). One of the two primers are labelled with a donor fluorophore (for example FAM), whereas the probe is labelled with a reporter fluorophore (for example Cy5). During each amplification cycle, the probe anneals to the extending primer, and energy transfer from the donor to the reporter is enabled due to the close proximity of the two (see Figure 5). The fluorescence emitted from the reporter depends directly on the amount of amplicons formed, which makes quantification possible (Rasmussen *et al.*, 2003; Hakhverdyan *et al.*, 2006).

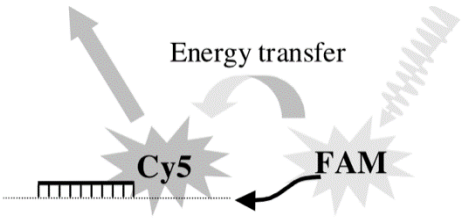

Figure 5: *An example of the PriProEt technique. One of the primers are labelled with a FAM (donor) fluorophore, while the probe is labelled with a Cy5 (reporter) fluorophore. After annealing, energy transfer from FAM (donor) to Cy5 (reporter) is enabled and fluorescence is emitted from the reporter* (Rasmussen *et al.*, 2003).

Another chemistry used is the *SYBR* Green I, which is a non-specific dye, that binds to the minor groove of double-stranded DNA. When bound to double-stranded DNA, it emits fluorescence that can be measured during the elongation phase of each cycle. The amount of fluorescence at any given time reflects the amount of double stranded DNA (Navarro *et al.*, 2015).

2.9.3.2.1 Basic principles of the quantitative real-time PCR

As previously mentioned, the PCR is carried out in a series of cycles, with three repeated phases. During the cycling the number of amplicons increases exponentially, reaching a plateau after varying number of cycles (see Figure 6) (Valasek and Repa, 2005). In the initial cycles of the qPCR, the fluorescent signal is weak which makes it impossible to distinguish from the background signal. When the number of amplicons increases during the PCR, the fluorescent signal produced will also increase, eventually reaching an arbitrarily placed threshold if the initial sample were positive (see Figure 6) (Kubista *et al.*, 2006). This is regardless of the chemistry used to elicit the fluorescent signal (Quinn *et al.*, 2011).

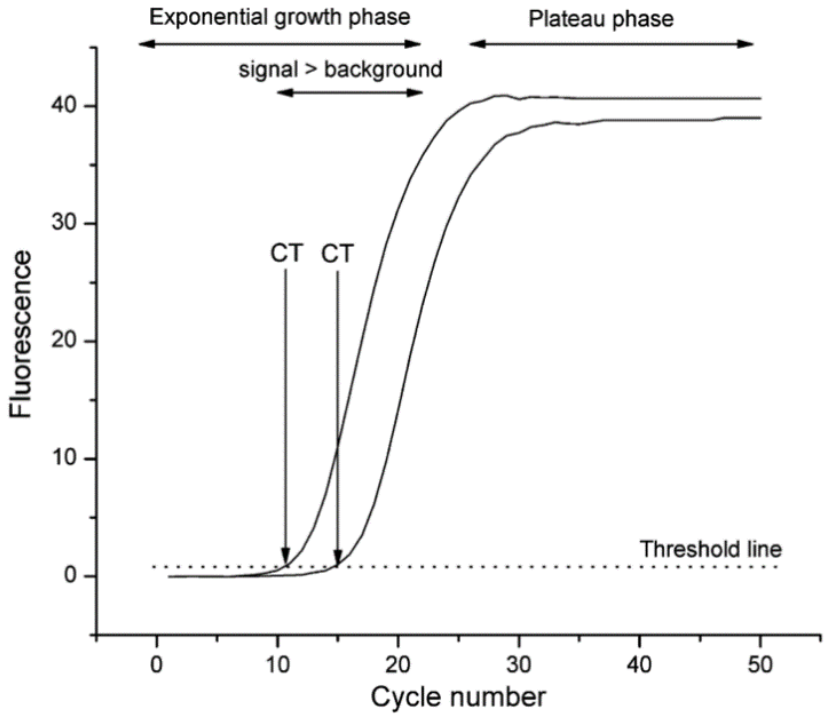

***Figure 6**: Response curve with shown threshold. The number of cycles required for the fluorescent signal to reach the threshold (Ct-value) is registered. The number of cycles required are inversely related to the amount of target DNA in the initial sample (Kubista, 2006).*

The number of cycles required to reach the threshold are called the Cycle Threshold value (Ct value (now known as the Cq-value)), and are inversely related to the amount of target DNA present in the initial sample (Kubista *et al.*, 2006; Schmittgen and Livak, 2008; Derveaux, Vandesompele and Hellemans, 2010). Thus, the lower the concentration of target DNA in the original sample, the higher the Ct value.

The PCR instrumentation detects the fluorescent signal emitted, and records the PCR progress. The PCR instruments must be able to provide a specific energy input for the excitation of the fluorescent

chemistries, and simultaneously detect the energy emitted at a specific wavelength (Valasek and Repa, 2005).

The results from the quantitative real-time PCR are displayed in an amplification curve, which is a graphic representation of the number of cycles as a function of the increase in the fluorescent signal (see Figure 7) (Valasek and Repa, 2005). For absolute quantification of target DNA in the original sample, a standard curve needs to be generated from a sample with known quantity of the target. The standard curve is generated by qPCR of a series of dilutions with known concentration of target DNA and are displayed as a function of the Ct values against the concentration of the target sequence. The standard curve is used for quantification of target DNA in the initial sample (Valasek and Repa, 2005).

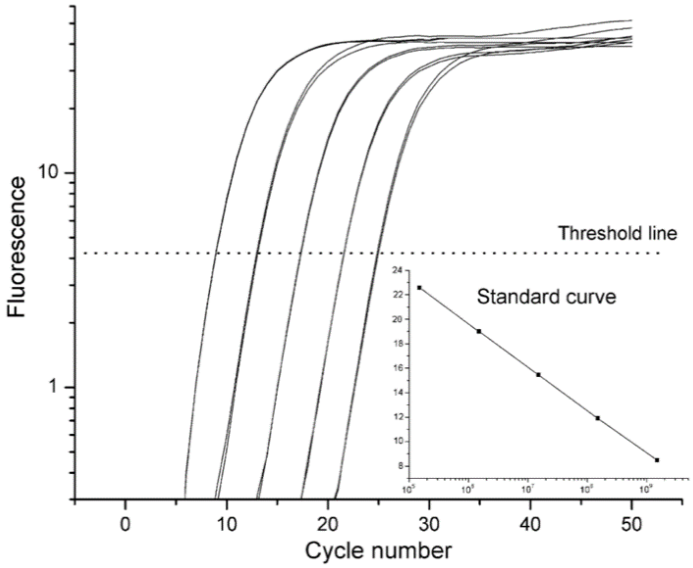

Figure 7: *Illustration of a response curve and a standard curve for five samples with known concentration of target sequence. The standard curve is constructed from the Ct-values obtained from the five response curves, which are plotted against the known concentrations (Kubista et al., 2006).*

During qPCR, a melting curve analysis can be obtained for assays using *SYBR green I* or *PriProET* chemistries (Navarro *et al.*, 2015). During the analysis, the temperature is gradually increased, and the fluorescence emission is monitored at each temperature step. When a temperature, which is specific for different assays, is reached denaturation occurs, thus showing a sharp drop in the

fluorescence signal due to dissociation of the probe (Valasek and Repa, 2005; Navarro *et al.*, 2015). The results are displayed in a dissociation curve (see Figure 8).

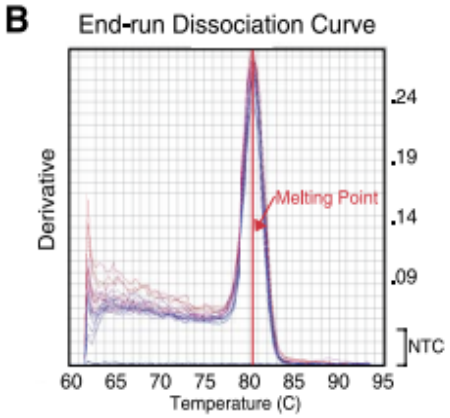

Figure 8: *Melt-peak analysis curve. At a specific temperature, denaturing occurs, thus showing a sharp drop in the fluorescent signal emitted (Valasek and Repa, 2005). The temperature where denaturing occurs is the meal peak.*

3 Research study

3.1 Background

PCV2 is often quantified by qPCR on pooled serum samples or penwise OF samples. Quantification of PCV2 viral load on a pen level using OF has gained some attention in recent years, because the collection procedure has some practical, economic and welfare advantages compared to collection of serum (Hernandez-Garcia *et al.*, 2017). Like OF collection, sock sampling offers some advantages compared to collection of serum, and might therefore be used as an alternative method for determining PCV2 viral load on a group level.

Correlation coefficients between PCV2 viral load in OF and PCV2 viral load in serum obtained in previous studies, have shown somewhat varying results. These estimated correlations were based on PCV2 viral load in OF from 20-30 pigs and PCV2 viral load in serum pools containing serum from four to five pigs or less (Kim, 2010; Oliver-Ferrando *et al.*, 2016; Nielsen, 2017). Therefore, the present study aimed to investigate whether a correlation between PCV2 viral load in OF and PCV2 viral load in serum existed, if the exact same pigs contributing to the OF sample by chewing the rope were included in the serum pool. Furthermore, it aimed to investigate whether a correlation between PCV2 viral load in faeces from sock samples and serum pools from the same pens existed.

Last, an assessment of the impact of individual pig's PCV2 viral load on a pooled serum sample were made.

3.2 Objectives and hypothesis

The present study had four main objectives.

Objective 1 was to investigate whether a correlation existed between PCV2 viral load in OF collected using a cotton rope and PCV2 viral load in a pooled serum sample from every pig in the pen in two different age groups.

Objective 2 was to investigate whether a correlation existed between PCV2 viral load in OF collected using a cotton rope and PCV2 viral load in a pooled serum sample from the pigs contributing to the OF sample by chewing the rope in two different age groups.

Objective 3 was to investigate whether a correlation existed between PCV2 viral load in faeces collected by sock samples and PCV2 viral load in a pooled serum sample from the pen in two different age groups.

Objective 4 was to examine the PCV2 viral load in individual serum samples, to assess the impact of an individual pig's PCV2 viral load on the serum pool results.

The null hypotheses were as follows:

H_0^1: The correlation coefficient between PCV2 viral load in OF and PCV2 viral load in pooled serum samples from the pens is equal to 0.

H_0^2: The correlation coefficient between PCV2 viral load in OF and PCV2 viral load in pooled serum samples from pigs contributing the OF sample is equal to 0.

H_0^3: The correlation coefficient between PCV2 load in faeces collected by sock samples and pooled serum samples is equal to 0.

H_0^4: The PCV2 viral load in individual serum samples have an equal impact on the PCV2 viral load in the serum pool.

4 Materials and methods

4.1 Study design

The study was carried out as a cross-sectional study over a period of three days. The study unit was the pen, and a total of 34 pens, divided into two age groups, were selected from one finisher herd in Northern Zealand.

In all pens, one sock sample, one OF sample, and blood samples from every pig in the pen were collected. Prior to collection of OF and blood samples, a number was sprayed on the back of every pig in the pen with livestock marking spray in order to make it possible to identify the individual pigs. The numbers on the pigs chewing the ropes were then noted in a schema during OF collection.

4.2 Selection of the study herd

The study herd was selected from the following inclusion criteria: A finisher herd with a positive PCV2 laboratory diagnosis at screening, and a pen size of minimum 15 pigs. The exclusion criterion was vaccination against PCV2.

To find herds for screening, all veterinary swine practices in Denmark were contacted by e-mail. Based on feedback, the first three herds meeting the inclusion criteria were selected for screening. One herd in Southern Jutland, one herd on Funen, and one herd on Zealand were selected. To increase the chances of finding two age groups positive for PCV2, five pigs in three different age groups were blood sampled. Serum from the five pigs in each age group were pooled and analysed by qPCR. The herd on Funen, and the herd on Zealand were PCV2 positive in all three age groups. The herd on Zealand was chosen as the study herd because it was located closest to DTU-VET. This allowed delivery of the collected samples at DTU-VET within 24 hours after collection, thereby minimizing the risk of pre-analytical errors due to inappropriate storage in the herd.

The study herd was a finisher herd with unknown SPF-health status. According to the farmer and our observations, no clinical signs of PMWS were present prior to and at the time of the sampling.

4.3 Sample size calculations

4.3.1 Sample size calculation for objective 1 and 2

Previous studies have reported correlation coefficients of 0.78, 0.69 and 0.39 between PCV2 viral load in OF samples and PCV2 viral load in pooled serum samples containing serum from four to five randomly chosen pigs from the same pen (Kim, 2010; Nielsen, 2017). Based on the previous reported correlations, along with economic considerations, an expected correlation coefficient of 0.63 was used to calculate the sample size for each age group. With a correlation coefficient of 0.63, a confidence level of 95% and a power of 80%, a sample size of 17 pens for each age group were calculated. The sample size calculations were conducted using the website www.sample-size.net.

4.3.2 Sample size calculation for objective 3

To the authors' knowledge, at the time of the present study, no previous studies regarding the correlation between PCV2 viral load faeces from sock samples and PCV2 viral load in serum samples have been conducted. Grau-Roma *et al.* (2009) reported a correlation coefficient of 0.72 between PCV2 viral load in faecal swabs and serum from individual pigs. Because of this, a high correlation coefficient was expected. For convenience, the same correlation coefficient as expected between OF and serum (0.63) was used to calculate the sample size. The calculation was performed as described in the previous section.

4.3.3 Sample size for objective 4

Due to economic restraints, only seven pens were selected for individual analysis.

4.4 Selection of study units

The study unit was the pen, and 17 pens were included for each age group. All pens were selected from one herd to keep as many factors as possible constant. First, the two age groups were selected based on the screening results and number of pigs in the pens. The youngest pigs (hereinafter designated as Age-group 1) were approximately 14-15 weeks old, and had entered the finisher site 2-3 weeks prior to sampling. Between 15 and 25 pigs were housed in each pen. The oldest pigs (hereinafter designated as Age-group 2) were approximately 18-19 weeks old, and had entered the

finisher site about 6-7 weeks prior to sampling. 16 pigs were housed in each pen, except in one pen where only 15 pigs were housed.

Secondly, within each age group, 17 pens out of a total of 24 pens were randomly selected using the website www.random.org. Three of the 17 pens within Age-group 1 and four of the 17 pens within Age-group 2 were similarly selected for analysis of individual serum samples from all the pigs in the pen. Within Age-group 1, pen 3, 10, and 14 were selected, while pen 1, 2, 11, and 17 were selected within Age-group 2. For both age groups, the 17 pens were distributed over 2 sections (see Figure 9).

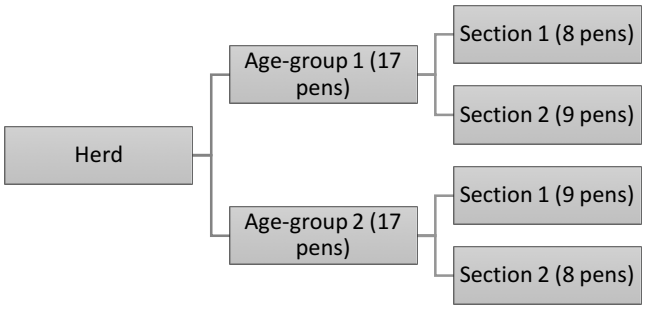

Figure 9: *Overview of the distribution of the pens in the two age groups.*

The screening of the study herd was carried out on the 7th of august 2017. The sampling was carried out two weeks later between the 21st and 23rd of august 2017.

The samples were collected in the following order: Sock samples, OF samples, blood samples. The sock samples were collected first to avoid stepping in the faecal material during the numbering of the pigs. OF was collected before the blood samples to minimize the risk of influencing the pigs' interaction with the ropes.

To minimize the time difference between sampling, the samples were collected within three days.

4.5 Sampling

4.5.1 Sock sampling

The sock samples were collected with a standard sock sample collection kit (Dianova, Lyngby, Denmark), containing a felt sock, a plastic sock, and a plastic bag. The collection was performed according to the manufacturers guidelines. In brief, the plastic sock was placed over the boots to avoid contamination from the boots. The felt sock was then placed over the plastic cover. To make the sock sample representative for the pen, the felt sock was in contact with the entire faecal-contaminated slatted floor area. After collection, the sock was placed in a plastic bag, which was marked with date, pen number, and age group. The samples were stored in a Styrofoam box with cooling elements until delivery at DTU-VET no later than 26 hours after collection. After delivery, the sock samples were kept refrigerated until centrifugation and purification maximum 24 hours after delivery.

4.5.2 Oral fluid sampling

All the samples were collected at least one hour after feeding, to avoid feed soiling of the ropes. Before collection of OF, a number was sprayed on the back of each pig in the pen. The rope was placed in shoulder height of the pigs on the inventory facing the aisle, which made it possible to observe the pigs at a distance. The numbers on the pigs chewing the rope were recorded in a schema (for schema used see appendix A). After 30 minutes, the ropes were evaluated for macroscopically visible faecal material, after which the ropes were wrung into individual plastic bags to release the OF. The OF was transferred from the plastic bag to a 15ml collection tube (Sarstedt, Denmark) and marked with date, pen number, and age group. The samples were stored in a Styrofoam box with cooling elements for a maximum of one hour before being frozen at -20 °C. During transportation, the samples were held frozen in a Styrofoam box with cooling elements, and at delivery at DTU-VET, stored at -20 °C for one week before centrifugation and purification.

4.5.3 Blood sampling

All the pigs in each pen were blood sampled. Blood was collected from the cranial vena cava in 10 ml serum vacutainers with clot activator (BD Vacutainer®, Kruuse, Denmark). The individual pig number along with pen number, age group and date were noted on the vacutainer. The individual animal number made it possible to identify the pigs that contributed to the OF samples. Between each blood sample, the needle was changed to avoid contamination from the previous blood sample. The storage before delivery and during transportation to DTU-VET was as described for the sock samples. After delivery, the blood samples were kept refrigerated until separation of serum, purification and pooling maximum 24 hours after delivery.

4.6 Laboratory analysis

4.6.1 Preparation of sock samples for qPCR analysis

The faecal sock samples with a known dry weight of the sock, were weighed and a calculated amount of phosphate-buffered saline (PBS) was added to obtain a 10% solution. The plastic bag containing the solution was processed in a Stomacher 400 (Seward, UK) for 2 minutes at 230 rpm, and 1 ml of the solution was transferred to a 2.0 ml microcentrifuge tube (Eppendorf, Denmark). Immediately before purification, a 5mm stainless-steel bead (QIAGEN, Denmark) was added to the Eppendorf tube containing the sample and processed in a TissueLyser II (QIAGEN, Denmark) for 3 minutes at 30 Hz. The sample was then centrifuged for 2 minutes at 10.000g and 350-400 µl supernatant was transferred to a new tube. Nucleic acids were purified on the QIASymphony extraction robot with

DSP virus/pathogen Mini Kit to extract the DNA. The protocol used was complex 200_v6_DSP. Each purification was run with a negative and positive control for every 22nd samples. The purified DNA was stored at - 20 °C until analysis.

4.6.2 Preparation of OF samples for qPCR analysis

After thawing of the OF samples, the samples were whirly mixed. 200 µl of the OF was transferred to a 2.0 ml microcentrifuge tube (Eppendorf, Denmark) and centrifuged for 3 minutes at 1000 rpm. 20 µl supernatant was added to a 2.0 ml microcentrifuge tube (Sarstedt, Denmark) containing 180 µl distilled DNase/RNase free water (Gibco, Denmark) to dilute the OF sample 1:10 prior to purification of DNA. The DNA purification was automated on a QIAcube (QIAGEN, Denmark) with QIAamp DNA Mini kit (QIAGEN) using the protocol Blood and body fluid spin protocol V3. A negative and a positive control were included for each run i.e. for every 10 samples. The purified DNA was stored at -20 °C until qPCR analysis.

4.6.3 Preparation of blood samples for qPCR analysis

The unstabilized blood samples were centrifuged at 3000 rpm for 10 minutes to separate the serum. After centrifugation, the serum was poured into a 3.6 ml cryotube (Sarstedt, Denmark). A pool containing 100 µl serum from all the pigs in the pen (in the following referred to as serum (all)) and a pool from all the pigs chewing the rope during OF collection (in the following referred to as serum (chewers)) was prepared for purification. From each pool, 200 µl was transferred to a QIAcube tube for DNA purification. The DNA purification was automated on a QIAcube (QIAGEN, Denmark) with protocol QIAamp DNA blood Mini kit with a negative and a positive control in each run i.e. for every 10 sample. The purified DNA was stored at -20 °C until PCR analysis.
For seven pens, individually serum samples from all the pigs in the pen were purified by the procedure described above.

4.6.4 qPCR analysis

All the samples were analysed at DTU-VET by quantitative real-time PCR with a detection limit of 10^3 PCV2 copies/ml and a quantification range for serum of $3.3*10^4$ – $3.3 * 10^9$ PCV2 copies/ml (Hjulsager *et al.*, 2009). Because of pre-extraction dilution the detection limit and quantification range for OF samples were $3.3*10^4$ and $3.3*10^5$- $3.3*10^9$ PCV2 copies/ml, respectively (Personal communication, Charlotte K. Hjulsager, DTU-VET). The detection limit for faeces were 10^4 PCV2

copies/g faeces and the quantification range was 10^5-10^{11} PCV2 copies/g faeces (Pedersen *et al.*, 2012).

For each reaction, two replicate reactions were prepared that contained three µl purified DNA and a PCR mixture consisting of 14.65 µl distilled DNase/RNase free water (Gibco, Denmark), 2.5 µl 10xPCR Gold buffer (Applied Biosystems, USA), 2.6 µl $MgCl_2$ [25 mM] (Applied Biosystems, Denmark), 0.5 µl of each dNTP [10 mM], 0.5 µl PCV2ORF1 forward primer [5 pmol/µl], 0.5 µl FAM-PCV2ORF1 reverse primer [15 pmol/µl], 0.5 µl PCV2-pro [25µM] (Cy5), and 0.25 µl Amplitaq Gold polymerase [5 U/µl] (Applied Biosystems, Denmark), resulting in a total volume of 25 µl. The primers and the probe used in the assay were selected based on a previous study (Ladekjær-Mikkelsen *et al.*, 2002). Their sequences are listed in Table 3. The reverse primer was labelled with a FAM donor fluorophore and the probe were labelled with a Cy5 reporter fluorophore.

Table 3*: Sequences of the reverse and forward primers and probe. The probe is labelled with a reporter fluorophore (Cy5), while the forward primer is labelled with a donor fluorophore (FAM).*

Virus	Primer	Sequence
PCV2	PCV2 ORF 1 forward primer	5´-GATGATCTACTGAGACTGTGTGA
	FAM-PCV2 ORF1 reverse primer	5´-6-FAM-AGAGCTTCTACAGCTGGGACA
	PCV2 specific probe	5´-TCAGACCCCGTTGGAATGGTACTCCTC-Cy5-3´

The qPCR was run on Rotor-gene Q (QIAGEN, Denmark) with the following profile: Denaturation at 95 °C for 10 minutes, 45 cycles of: 95 °C for 15s, 60 °C for 40s, 75 °C for 20 seconds. This was followed by 5 minutes of 75 °C, 30s of 95 °C, and 1 minute of 45 °C. A melt peak analysis was obtained by increasing the temperature from 45 °C to 99 °C by one degree pr. step. The fluorescent signal was collected during the annealing step of each amplification cycle and during each step of the melt peak analysis. The FAM flourophore on the reverse primers was excited at 470 nm and the fluorescent signal from the Cy5 was emitted and collected at 660nm. The Ct threshold was set to 0.02 in the exponential phase of the amplification and the NTC threshold to 10%. The events behind the emission of fluorescence are described in section 2.9.3.2.

All the samples were run in duplicates including a negative no template control (NTC) (distilled DNase/RNase free water (Gibco, Denmark)) and a positive control containing 10^6 PCV2 copies/ml. A sample was considered positive if the PCV2 viral load exceeded 10^3 PCV2 copies/ml serum or 10^4

PCV2 copies/ml OF, and only when a Ct value, plus a melt peak fairly similar to the positive control, was obtained for both duplicates. The melt peak for PCV2 is around 72 °C. A sample was considered negative if the Ct value was ≥37 and the quantitative result was below the detection limit. For approval of the PCR run, the negative controls had to be without a Ct value and a positive melt peak.
The PCV2 viral load in serum and OF were quantified from a standard curve as previously described by Hjulsager *et al.* (2009). In brief, the standard curve was made from running qPCR on dilutions prepared in 100 ng/µl yeast tRNA (Ambion) of a PCV2 plasmid. This plasmid was constructed by cloning the PCV2 PCR product into a plasmid vector. The concentration of the plasmid was measured spectrophotometrically (Hjulsager *et al.*, 2009). In each qPCR run, a positive control of 10^6 plasmid copies of PCV2/reaction was included and used as a fix point for import of the standard curve. The PCR efficiency was 90-100% measured from the standards (Hjulsager *et al.*, 2009).
The PCV2 viral load in faeces was quantified similarly from a standard curve prepared from dilutions of PCV2 virus in 10% faeces. The PCR efficiency was 83% measured from the standards (Personal communication, Charlotte K. Hjulsager, DTU-VET).

4.7 Statistical analysis

Data Analyses were carried out using JMP® version 13.0 (SAS Institute Inc., 2016).
All the PCV2 viral loads were analysed on a log-transformed scale except for calculation of the arithmetic mean and median from individual serum samples, which was calculated from the original data and then log transformed.
All the log transformed PCV2 viral loads were tested for normal distributions from histograms and normal quantile plots. For normal distributed variables, parametric analyses were carried out.
Descriptive statistics consisted of graphical illustrations and summary statistics such as arithmetic mean and median.
To test for differences in the mean PCV2 viral load between different sample types within each age group, paired t-tests were conducted using the matched pairs function in JMP®. To test for differences in the mean PCV2 viral load between different sample types between age groups, t-tests were performed using the fit Y by X function in JMP ®. Prior to performing the tests, variances of the groups were tested using an F-test. When the variances were not equal i.e. significantly different between the groups, an unequal variance t-test was performed.
To estimate the degree of linear association between PCV2 viral load in OF and serum and between faeces from sock samples and serum, a Pearson's product moment correlation coefficient (denoted r)

was calculated using the Multivariate function in JMP ®. Because previous studies have reported a Spearman's rank correlation coefficient (denoted ρ), a Spearman's correlation coefficient was also calculated and reported, but for comparative reasons only. The correlation coefficients were interpreted as shown Table 4.

***Table 4**: Catorgization for interpretation of correlations coefficients (Modified from Mukaka, 2012)*

Size of correlation	Interpretation
0.9 - 1.0 (-0.90 to - 1.0)	Very high positive (negative) correlation
0.7 - 0.9 (- 0.7 to - 0.9)	High positive (negative) correlation
0.5 - 0.7 (-0.5 to -0.7)	Moderate positive (negative) correlation
0.3-0.5 (-0.3 to -0.5)	Low positive (negative) correlation
0.0 -0.3 (0.0 to - 0.3)	No correlation

Separately for each age-group, the association of number of pigs in the pen, and number of pigs that chewed the rope on the PCV2 viral load in OF, were tested using multiple linear regression analysis with Fit model function JMP ®. The PCV2 viral load in serum (all) or serum (chewers) were included as independent variables, while 'number of pigs in the pen' and 'number of pigs chewing the rope' were considered as controlling variables. Similarly, the association of number of pigs in the pen on the PCV2 viral load in faeces from sock samples was tested. The PCV2 viral load in serum (all) was included as independent variable, while 'number of pigs in the pen' was considered as controlling variable. In the analysis 'number of pigs in the pen' and 'number of pigs chewing the rope' were included as categorical variables, categorized from the 33rd percentile, as "high", "medium" and "low". The significance of each of the variables were interpreted from an F-test. When running the models, the variables were excluded one by one, if they were not statistically associated with the PCV2 viral load in OF or faeces ($p > 0.05$). Residuals were tested for normal distribution from normal quantile plots.

The statistical analyses were interpreted with a significance level set at $p < 0.05$.

5 Results

5.1 Sampling

Samples were collected from Age-group 1 (14-15 weeks of age) and Age-group 2 (18-19 weeks of age) in three days. Due to the time pressure, samples from Age-group 1, section 2 were collected

during the night. The dates and time of the sample collection from each section in the two age groups are summarized in Figure 10.

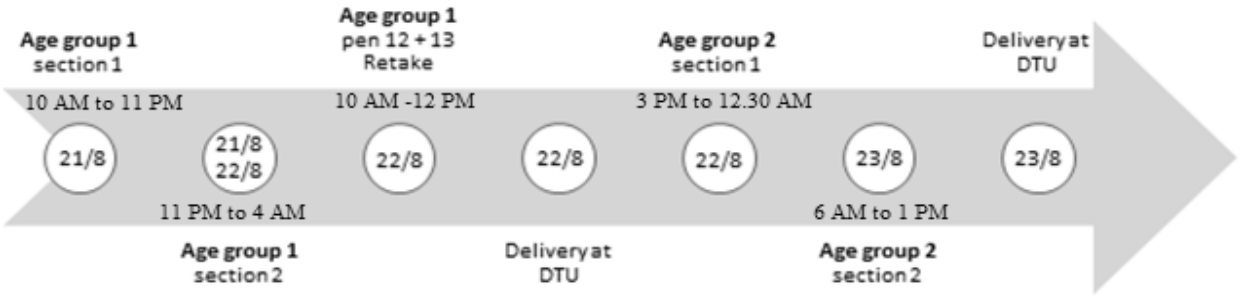

Figure 10: *Timeline for sample collection. All the samples in Age-group 1 were collected over a period of 18 hours and in Age-group 2 over a period of 21 hours. Notice that samples from Age-group 1, section 2 were collected during the night.*

In total, 34 sock samples, 34 OF samples, and 639 blood samples were collected. As previously mentioned, two serum pools from each pen were assembled in the laboratory: A pool of all the pigs in the pen (serum (all)) and a pool of all the pigs that chewed the rope during OF collection (serum (chewers)).

The pigs were exposed to the rope for 30 minutes, except in pen 8 and 9, Age-group 2, where the pigs were exposed to the rope for only 24 and 28 minutes, respectively, due to earlier feeding than expected.

The mean percentage of pigs that chewed the rope was 76.6 % [56.2 % - 100 %] and 77.6 % [62.5 % - 100%] for Age-group 1 and Age-group 2, respectively. The mean percentage of pigs that chewed the rope during collection of OF at night and during day was 77.1 % and 75.9 % respectively.

5.2 Effect of age and time of collection on the percentage of pigs that chewed the rope

The effects of age and time of collection on the percentage of pigs that chewed the rope, were analysed using a student's t-test. Neither age group nor the time of collection of OF had an impact on the percentage of pigs that chewed the rope in the present study (night vs. day $p = 0.85$, Age-group 1 vs Age-group 2: $p = 0.77$).

5.3 Presence of faecal material on the ropes

The presence of macroscopically visible faecal material on the ropes was assessed after 30 minutes of exposure to the pigs. Apparently, no macroscopically visible faecal material was evident on the ropes.

5.4 Overview of PCV2 viral load in serum pools, oral fluid, and faecal sock samples

All the samples were positive for PCV2. The raw data of the PCV2 viral load in the different samples are listed in appendix B.1, B.2 and B.3. The distributions of the PCV2 viral load in serum (all), serum (chewers), OF, and faeces from sock samples in the two age groups are illustrated in Figure 11.

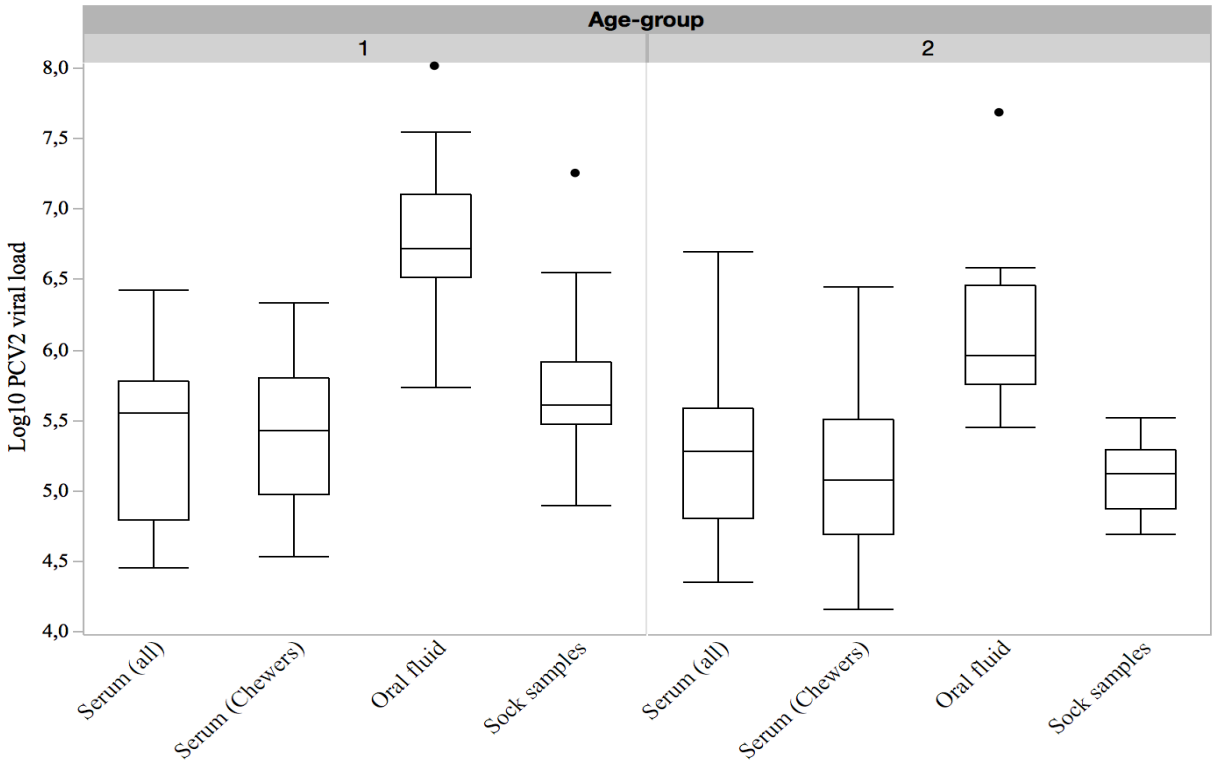

Figure 11: *The distributions of the PCV2 viral load in serum (all), serum (chewers), OF and faeces from sock samples in Age-group 1 (left) and Age-group 2 (right). The points outside the boxplots illustrate potential outliers which is pens with a PCV2 viral load higher than 1.5* interquartile range.*

No statistically significant difference was observed between the mean PCV2 viral load in serum (all) and serum (chewers) neither in Age-group 1 (p = 0.99) nor in Age-group 2 (p = 0.78). The difference between PCV2 viral load in the matched serum (all) and serum (chewers) was <1 $\log_{10}$ PCV2 copies/ml serum. Furthermore, no statistically significant difference was observed in the mean PCV2 viral load in serum (all) (p = 0.66) or the mean PCV2 viral load in serum (chewers) (p = 0.36) between age groups.

A significantly higher PCV2 viral load in OF compared to PCV2 viral load in serum (chewers) and serum (all) was evident (p <0.0001). The mean difference between PCV2 viral load in OF and PCV2 viral load in serum (chewers) was 1.36 $\log_{10}$/ml serum [1.10;1.64] and 0.92 $\log_{10}$/ml serum [0.53;1.32] in Age-group 1 and Age-group 2, respectively. Furthermore, a higher and statistically significant PCV2 viral load in OF (p = 0.002) and in faeces (p = 0.0002) was observed in Age-group 1 compared to Age-group 2.

5.5 Association between serum, OF, and faeces in sock samples

In the following sections, associations between PCV2 viral load in serum pools, OF, and faeces from sock samples are illustrated from scatterplots with fitted regression lines. The correlation coefficients are furthermore reported.

5.5.1 Association of number of pigs in the pen and number of pigs that chewed the rope on PCV2 viral load in oral fluid and faeces from sock samples

It was investigated if 'number of pigs in the pen' and 'number of pigs chewing the rope' were significantly associated with the PCV2 viral load in OF or faeces sock samples in either of the two age groups, when included in a model with serum (all) or serum (chewers). However, none of the two variables were significantly associated with the PCV2 viral load in OF or sock samples in neither Age-group 1 nor in Age-group 2. The correlation coefficients between serum and OF, and serum and faeces were therefore calculated without controlling for number of pigs in the pen and number of pigs chewing the rope.

5.5.2 Association between PCV2 viral load in OF and PCV2 load in pooled serum (all)

The scatterplots of the PCV2 viral load in OF against the PCV2 viral load in serum (all) are illustrated in Figure 12.

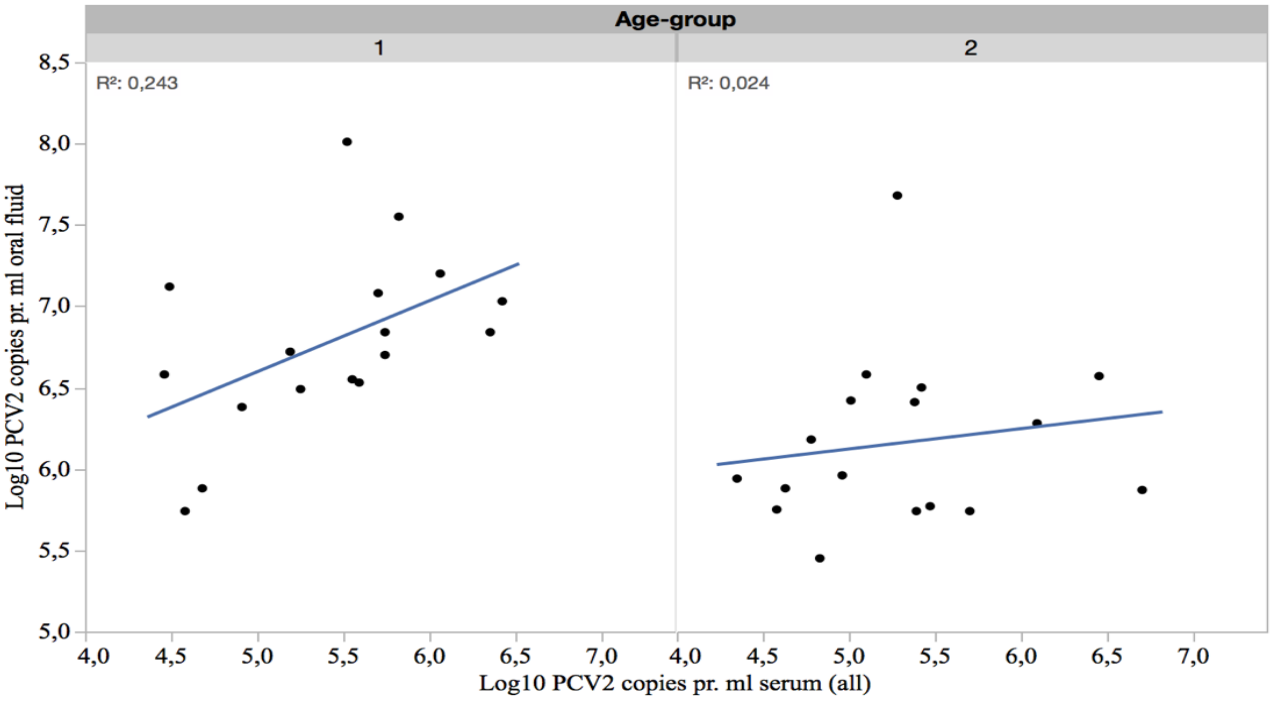

Figure 12: *Scatterplots of the PCV2 viral load in OF against the PCV2 viral load in serum (all) with fitted regression lines. Left: Age-group 1. Right: Age-group 2.*

Correlation coefficients with corresponding p-values are listed in Table 5.

Table 5: *Correlation coefficients with 95% confidence intervals and corresponding p-values for Age-group 1 and Age-group 2. In bold, Pearsons correlation coefficients (r). Spearmans correlation coefficients (ρ) are listed for comparability. *Indicates statistically significant correlations (p <0.05)*

	Age-group 1		Age-group 2	
Variables	**Correlation**	**P-value**	**Correlation**	**p-value**
Log10 PCV2 copies pr. ml OF vs. Log10 PCV2 copies pr. ml serum (all)	**r = 0.50** [0.02;0.8] ρ = 0.50	**0.045*** 0.044*	**r = 0.16** [-0.4;0.6] ρ = 0.09	**0.64** 0.72

In Age-group 1, a significant moderate correlation between PCV2 viral load in OF and PCV2 viral load in serum (all) was observed (see Table 5). No correlation was observed for Age-group 2 (see Table 5). Hypothesis H_0^1 was therefore rejected in Age-group 1, but accepted in Age-group 2.
From the scatterplot, one pen with a high PCV2 viral load in OF was evident for Age-group 2.
The correlation coefficient increased but did not become statistically significant, when the pen was removed from the data set (r = 0.25, p = 0.35). Thus, a linear association between PCV2 viral load in OF and PCV2 viral load in serum (all) was observed for Age-group 1, but not for Age-group 2.

5.5.3 Association between PCV2 viral load in OF and PCV2 viral load in serum (chewers)

The scatterplots of the PCV2 viral load in OF against the PCV2 viral load in serum (chewers) are illustrated in Figure 13.

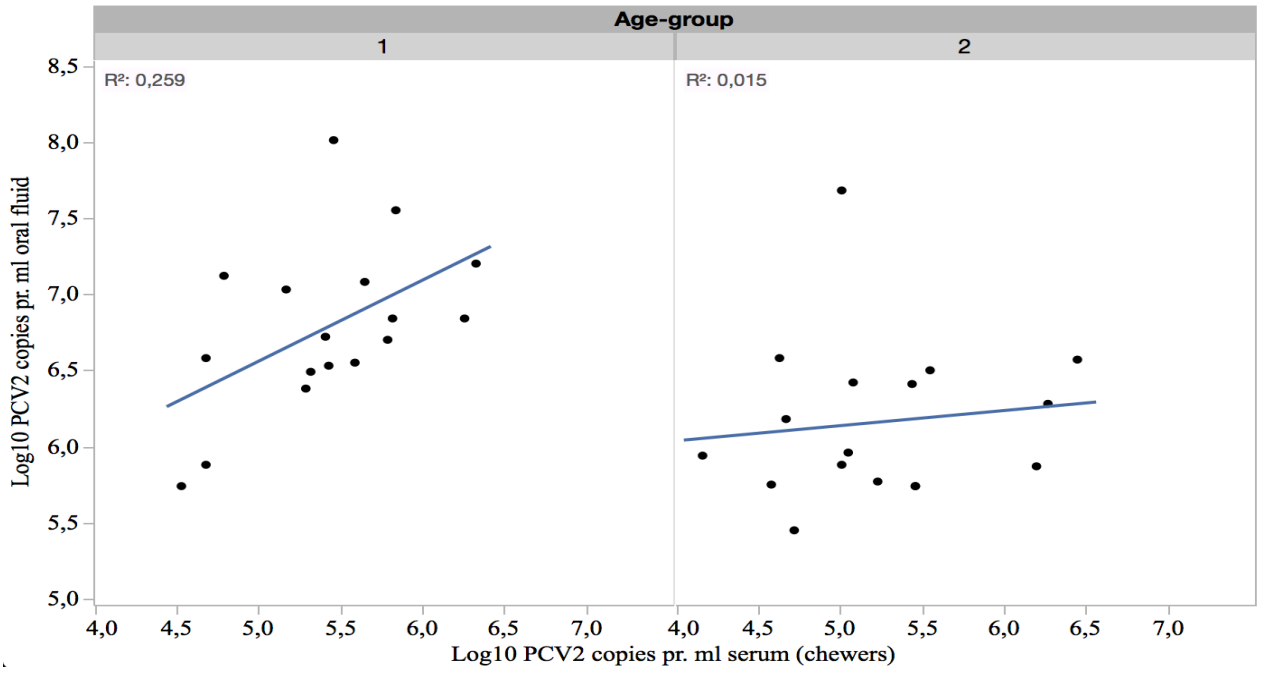

Figure 13: *Scatterplots of the PCV2 viral load in OF against the PCV2 viral load in serum (chewers) with regression lines. Left: Age-group 1. Right: Age-group 2.*

Correlation coefficients with corresponding p-values are listed in Table 6.

Table 6: *Correlation coefficients with 95% confidence intervals and corresponding p-values for Age-group 1 and Age-group 2. In bold, Pearsons correlation coefficients (r). Spearmans correlation coefficients (ρ) are listed for comparability. *Indicates statistical significant correlations (p-value <0.05).*

	Age-group 1		**Age-group 2**	
Variable	**Correlation**	**p-value**	**Correlation**	**p-value**
Log10 PCV2 copies/ml OF vs. Log10 PCV2 copies/ml serum (Chewers)	**r = 0.51** [0.04;0.8] ρ = 0.57	**0.04*** 0.02*	**r = 0.12** [-0.4;0.7] ρ = 0.13	**0.55** 0.62

A moderate, positive, and statistically significant correlation between PCV2 load in OF and PCV2 load in serum (chewers) was observed in Age-group 1 (see Table 6). No correlation was observed for Age-group 2 (see Table 6). Hypothesis H_0[2] was therefore rejected in Age-group 1, but accepted in Age-group 2. Removal of the pen with a high PCV2 viral load in OF in Age-group 2, increased the correlation coefficient, but it did not become statistically significant (r = 0.28, p = 0.27).

Thus, a linear association between PCV2 viral load in OF and serum (chewers) was observed for Age-group 1, but not for Age-group 2.

5.5.4 Association between PCV2 viral load in serum (all) and PCV2 viral load in faeces

The scatterplots of the PCV2 viral load in faeces against the PCV2 viral load in serum (all) are illustrated in Figure 14.

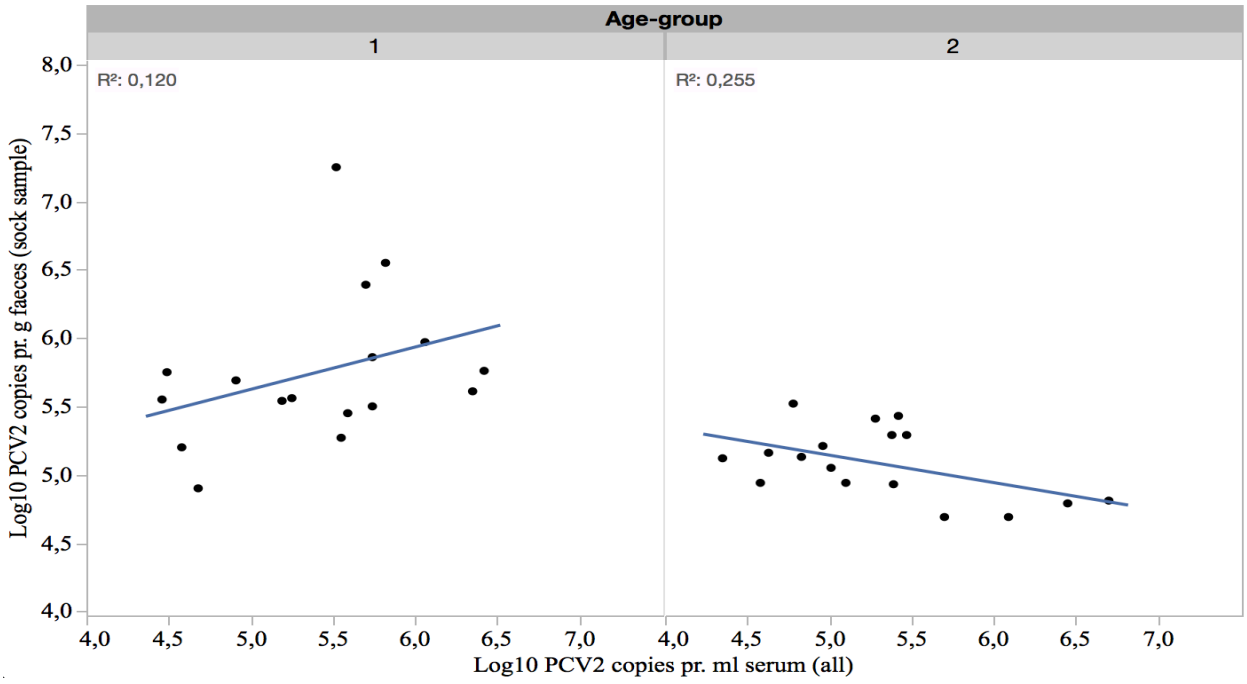

Figure 14: *Scatterplots of the PCV2 viral load pr. g faeces from sock samples against the PCV2 viral load pr. ml serum (all) with regression lines. Left: Age-group 1. Right: Age-group 2.*

Correlation coefficients with corresponding p-values are listed in Table 7.

Table 7: *Correlation coefficients with 95% confidence interval and corresponding p-values for Age-group 1 and Age-group 2. In bold, Pearsons correlation coefficients (r). Spearmans correlation coefficients (ρ) are listed for comparability. *Indicates statistical significant correlations (p-value <0.05).*

	Age-group 1		**Age-group 2**	
Variable	**Correlation**	**p-value**	**Correlation**	**p-value**
Log10 PCV2 copies/serum (all) vs. log10 PCV2 copies/g faeces	**r = 0.35** [-0.2;0.7] ρ = 0.44	**0.17** 0.08	**r = -0.51** [-0.8;-0.03] ρ = -0.4	**0.04*** 0.09

No statistically significant correlation was observed for Age-group 1 (see Table 7), while a moderate, negative, and statistically significant correlation was observed for Age-group 2 (see Table 7). Hypothesis H_0^3 was therefore accepted in Age-group 1, but rejected in Age-group 2.

From the scatterplots, one pen in Age-group 1 had a high PCV2 viral load in faeces. When the pen was removed from the dataset, the correlation coefficient increased but did not become statistically significant (r = 0.44, p = 0.1).

In summary, while no statistically significant correlation was found in Age-group 1, a statistically significant, negative correlation was found in Age-group 2.

5.6 PCV2 viral load in serum from individual animals in seven randomly chosen pens

The PCV2 viral load in serum from each pig in seven pens with reference lines set at the $\log_{10}$ PCV2 viral load in serum (all) (red line), $\log_{10}$ PCV2 viral load in OF (blue line), and $\log_{10}$ PCV2 viral load in faeces (yellow line) are illustrated in Figure 15 and Figure 16.

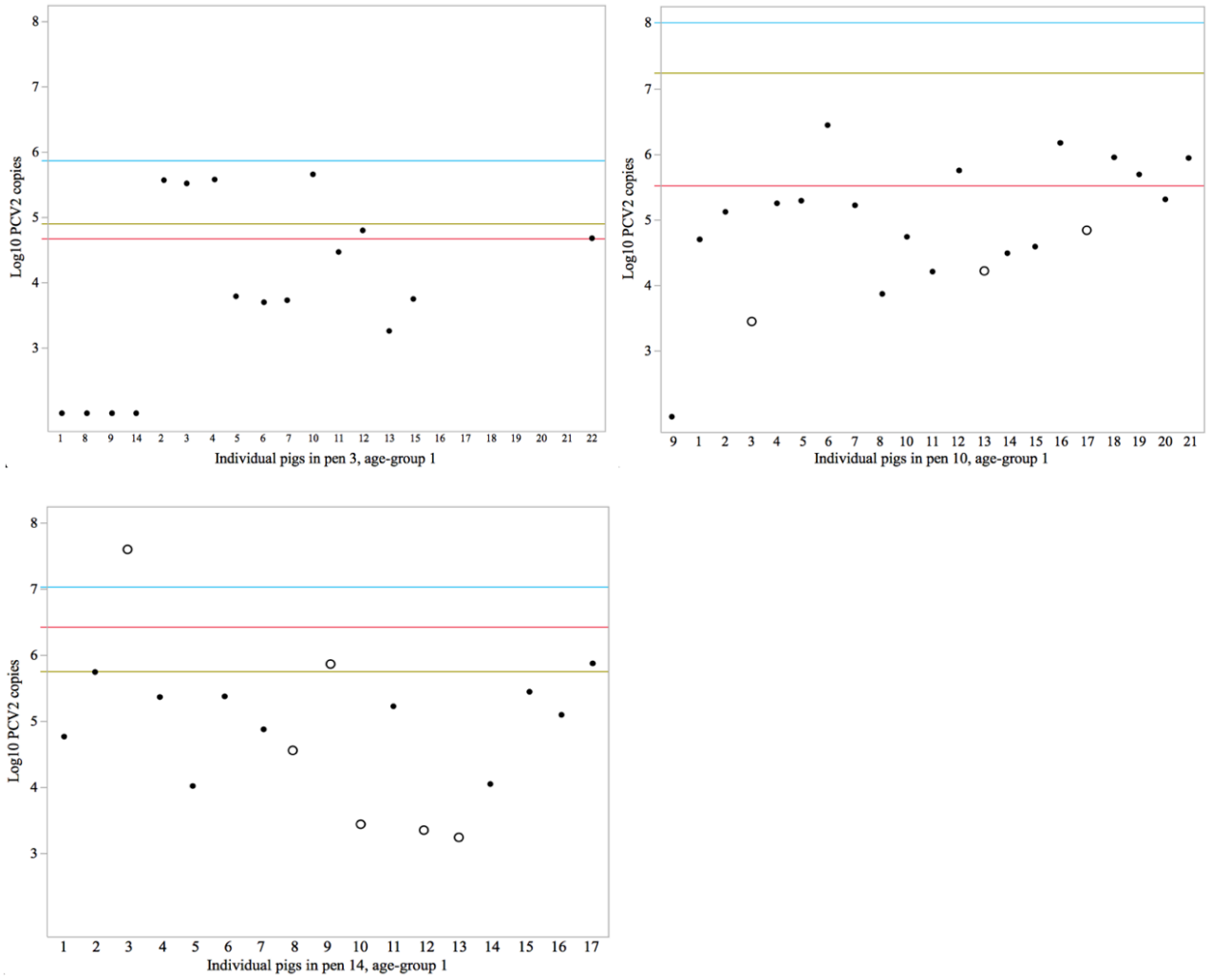

Figure 15: *The PCV2 viral load for each of the pigs in the pens in Age-group 1 with reference lines set at the $\log_{10}$ PCV2 viral load pr. ml serum (all) (red line), $\log_{10}$ PCV2 viral load pr. ml OF (blue line), and $\log_{10}$ PCV2 viral load pr. g faeces (yellow line). Black dots symbolize pigs chewing the rope, and clear dots symbolize pigs that did not chew the rope during OF collection.*

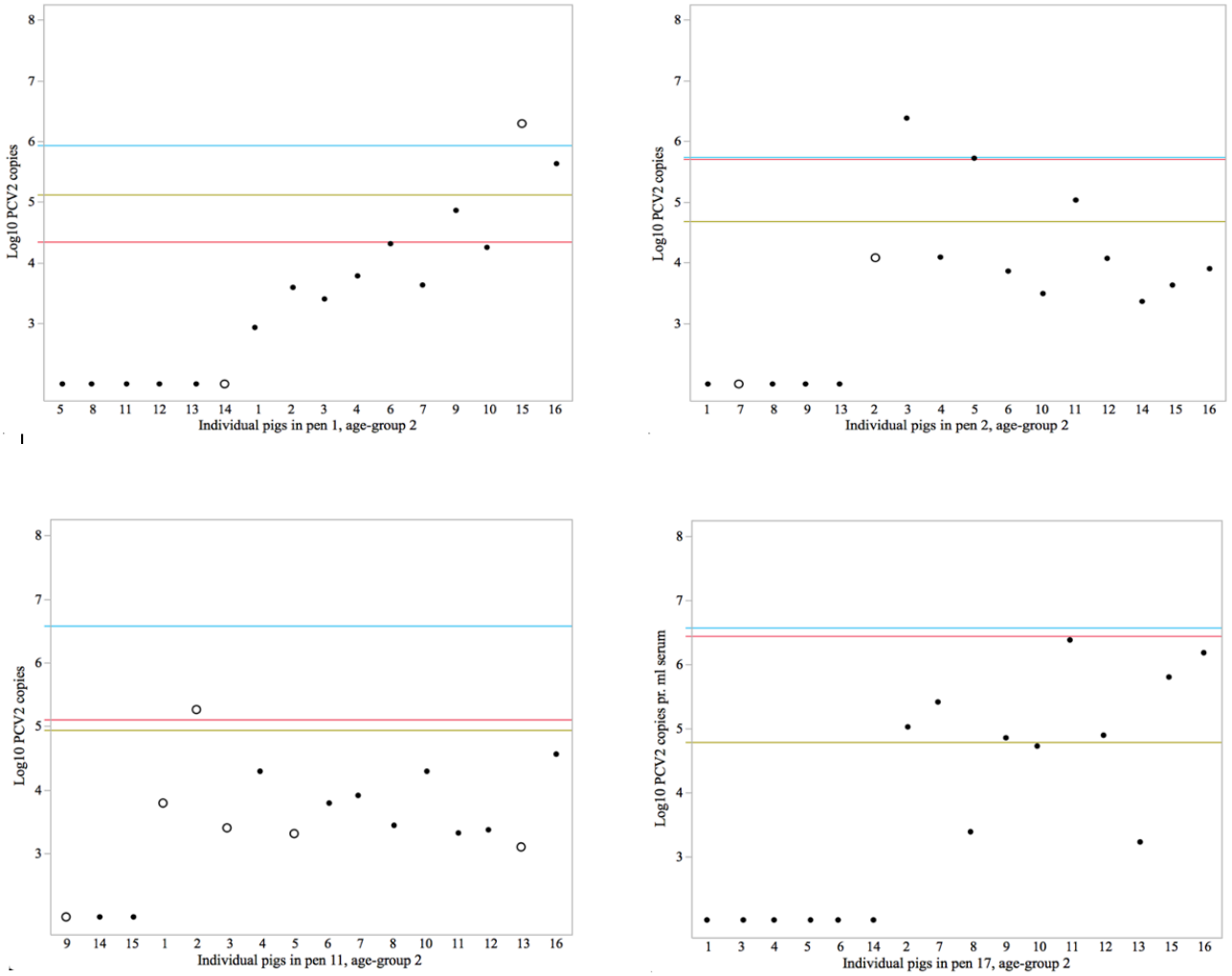

Figure 16: *The PCV2 viral load for each of the pigs in the pens in Age-group 2 with reference lines set at the log_{10} PCV2 viral load pr. ml serum (all) (red line), log_{10} PCV2 viral load pr. ml OF (blue line), and log_{10} PCV2 viral load pr. g faeces in sock samples (yellow line). Black dots symbolize pigs that chewed the rope, and clear dots symbolize pigs that did not chew the rope during OF collection.*

A great variation in the serum PCV2 viral load in individual pigs within and between pens was observed. Overall, more pigs had an individual PCV2 viral load below than above the PCV2 viral load in serum (all). For example, only one pig had a PCV2 viral load as high as the PCV2 viral load in the matched serum (all) in pen 14 (Age-group 1), pen 11, and pen 17 (Age-group 2). In pen 2, Age-group 2, only two pigs had a PCV2 viral load as high as the matched serum (all). Furthermore, it was a bit peculiar that in pen 3, 10, and 11, none of the pigs that chewed the rope had a serum PCV2 viral load as high as the PCV2 viral load in OF.

From the plots, it was clarified that there was no consistent difference between the PCV2 viral load in serum and OF, or between the PCV2 viral load in serum and faeces from sock samples.

The PCV2 viral load in serum (all), the mean PCV2 viral load, and median PCV2 viral load calculated from individual pigs are listed in Table 8.

Table 8: *PCV2 viral load in serum (all) by qPCR and the calculated arithmetic mean and median PCV2 viral load based on individual pigs' PCV2 viral load for seven different pens.*

Pen	Serum (all) log10 PCV2 copies/ml serum	Mean of individual pigs log10 PCV2 copies/ ml serum	Median of individual pigs log10 PCV2 copies/ml serum
3, age 1	4.68	5.04	3.68
10, age 1	5.52	5.45	5.01
14, age 1	6.42	6.40	5.08
1, age 2	4.35	5.19	3.51
2, age 2	5.7	5.29	3.75
11, age 2	5.1	4.26	3.42
17, age 2	6.45	5.5	4.43

The PCV2 viral load in serum (all) was relatively close to the arithmetic mean calculated from the individual PCV2 viral load in each pig (difference < 1 $\log_{10}$ PCV2 copies/ml serum), but relatively far from the median PCV2 viral load in four out of seven pens (difference ≥1 $\log_{10}$ PCV2 copies/ml serum).

Thus, the PCV2 viral load in serum (all) and the arithmetic mean were relatively close to each other, while relatively far from the median. The variation within the pen varied greatly with a large proportion of pigs with an individual PCV2 viral load in serum below the PCV2 load in serum (all).

5.7 Within pen prevalence

The within-pen prevalence for the seven pens is listed in Table 9.

Table 9*: Within pen prevalence with 95% confidence intervals calculated from three pens in Age-group 1 and four pens in Age-group 2. 1 refers to Age-group 1, [2] refers to Age-group 2*

Pen [Age-group]	# positive pigs	# negative pigs	Within-pen prevalence	95% CI
3 [1]	11	4	73.3%	[51.0%;95.7%]
10 [1]	20	1	95.2%	[86.1%;100%]
14 [1]	17	0	100%	-
1 [2]	10	6	62.5%	[38.8%;86.2%]
2 [2]	11	5	68.8%	[46.0%;91.5%]
11 [2]	13	3	81.3%	[62.1%;100%]
17 [2]	10	6	62.5%	[38.8%;86.2%]

Overall, an apparent higher within pen prevalence was evident in Age-group 1 compared to Age-group 2.

6 Discussion

6.1 Key results

In the present study, it was investigated whether a correlation between PCV2 viral load in serum pools, OF samples and faeces from sock samples existed. The results revealed that no strong correlations between PCV2 viral load in serum pools and OF, or between PCV2 viral load in serum pools and faeces from sock samples existed.

Individual samples from pigs in the pen revealed that in four out of seven pens, only one or two pigs had a PCV2 viral load as high as the PCV2 viral load in serum (all), and that serum (all) were ≥ 1 $\log_{10}$ higher than the calculated median in five out of seven pens.

Another interesting finding in the present study, was that the PCV2 viral load in OF was higher than the PCV2 viral load in pooled serum samples, with a mean difference of 1.36 and 0.92 $\log_{10}$ PCV2 copies/ml in Age-group 1 (14-15 weeks of age) and Age-group 2 (18-19 weeks of age) respectively.

6.2 Strength and limitations of the study design

The sample size calculations were based on an expected correlation coefficient of 0.63. However, some of the calculated correlation coefficients in the present study were far from the expected correlations. Therefore, the non-significant correlation coefficients obtained in the present study might merely be due to a too small sample size.

Due to time constraints and economic reasons, only 34 pens divided into two age groups were included in the study. To strengthen the internal validity of the study, all 34 pens were sampled in one finisher herd. However, this lowered the external validity, and whether the results obtained in the present study apply to other herds and age groups, therefore, need further investigation.

Also due to time constraints, some of the samples were collected in the evening and during the night. Since sampling during the night is not common practice and it might be expected that the pigs exhibit lower activity during the night than during the day, it was speculated if the time of collection had an influence on the proportion of pigs that chewed the rope. However, no difference was found between the proportion of pigs that chewed the rope at night and during the day. In addition, the mean percentage of pigs that chewed the rope was 76.6% [56.2% - 100%] and 77.6% [62.5% - 100%] for Age-group 1 and Age-group 2, respectively.

After collection, the OF samples were kept at -20 °C before delivery to DTU and kept frozen during transportation in a Styrofoam box with cooling elements. Previous studies regarding PCV2 in OF have reported that the OF samples were stored at -80 °C before and after purification. However, studies on the stability of viral RNA in OF have shown that viral RNA is relatively resistant

to degradation at -20 °C (Prickett et al., 2010; Jones and Muehlhauser, 2014). Because RNA is considered less stable than DNA (Steinhauer and Holland, 1987), the storage conditions at -20 °C in the present study is not expected to have had an essential impact on the results.

Nielsen (2017) suggested that a higher correlation between PCV2 viral load in serum and PCV2 viral load in OF may be expected when the proportion of pigs contributing to the serum sample approaches the proportion of pigs contributing to the OF sample. To investigate this, serum pools containing up to 25 pigs were made. It should be kept in mind that serum pools of these sizes have not been validated, and are rarely investigated in veterinary practices.

Some of the individual serum samples were classified as negative, since the PCV2 viral load was below the detection limit of 10^3 PCV2 copies/ml serum. However, the negative pigs might have had a PCV2 viral load just below the detection limit rather than 0 PCV2 copies/ml. If this is true, it might have falsely lowered the arithmetic mean, but because of a relatively low number of negative pigs, this has most likely not been of importance in the present study.

6.3 PCV2 viral load in oral fluid samples, faecal sock samples, and serum pools

An interesting finding in the present study, was that a higher PCV2 viral load in OF compared to the matched serum (all) and serum (chewers) was observed for both age groups in most pens. In addition, from Figure 15 and 16 it was observed that in three out of seven pens, none of the pigs that chewed the rope had a serum PCV2 viral load as high as the PCV2 load in OF.

That the PCV2 viral load is higher in OF samples and oral/nasal swabs compared to serum pools and individual serum samples seems to be supported by other studies (Patterson *et al.*, 2011; Oliver-Ferrando *et al.*, 2016; Nielsen, 2017).

It has been suggested that the higher PCV2 viral load in OF might be due to 1) spoiling of the ropes with traces of faeces, as pigs supposedly could deposit faecal material from the mouth/skin along with OF when chewing the rope, or 2) that pigs with a high viral load is included in the OF sample, but not the serum sample, when serum samples only contain serum from a few pigs (Oliver-Ferrando *et al.*, 2016). These suggestions could not be supported in the present study, since none of the ropes were spoiled with macroscopically visible faecal material, which reduces the likelihood that PCV2 in faeces could explain the higher PCV2 viral load in OF. Furthermore, serum (all) contained all the pigs in the pen and pigs with a high viral load was therefore included in both sample types, but the PCV2 viral load in OF was still higher than the PCV2 viral load in serum pools.

Alternatively, the explanation for the higher PCV2 viral load in OF, may be biological factors such as increased shedding when chewing, or initial replication of PCV2 in the tonsil, which has previously been suggested (Oliver-Ferrando *et al.*, 2016).
With this being said, comparison of the absolute PCV2 copy number in OF and serum might be difficult for the sole reason that it is different sample materials.
Whatever the reason for the higher PCV2 viral load in OF compared to serum, it is important to keep this in mind when interpreting the results. To the authors' knowledge, this is not being taken into consideration in Danish veterinary practices, which, based on the results from the present study, often causes the viral load in the pens to be overestimated. However, it should again be emphasized that because only one finisher herd was included in the present study and only few other studies have addressed this, it is not known whether this is a general tendency.

In the present study, a higher PCV2 viral load in OF and faeces from sock samples was evident for Age-group 1 compared to Age-group 2. If it is assumed that the pigs in Age-group 2 are further along in the course of infection, it is not entirely unexpected that the PCV2 viral load decreases from Age-group 1 to Age-group 2, as it has been illustrated that a decrease in both PCV2 viral load in faeces and OF/oral swabs occur over time (Patterson *et al.*, 2011; Prickett *et al.*, 2011; Oliver-Ferrando *et al.*, 2016). However, this usually coincides with a decrease in the PCV2 viral load in serum samples (Patterson *et al.*, 2011; Oliver-Ferrando *et al.*, 2016), which was not the case in serum pools in the present study. One of the main differences in the present study compared to previous studies was the difference in the size of the serum pools investigated. A previous study showed that from a theoretical pool of five pigs, one pig could potentially dominate the pool leading to a higher pool result (Nielsen, 2017). A large pool size will increase the probability of including pigs with a high PCV2 viral load, thus potentially increasing the serum pool result. This might explain the absence of a similar lower load in serum. This assumption is supported by the fact that an apparent lower PCV2 viral load in individual serum samples, and a lower within-pen prevalence was observed in Age-group 2 compared to Age-group 1, but still with few pigs having an apparent high PCV2 viral load in serum. However, it should be emphasized that due to the cross-sectional nature of the present study, the infection dynamic in this herd was not known, and the lower PCV2 viral load in OF, faeces and in individual serum samples in Age-group 2 compared to Age-group 1 might therefore be purely coincidental.

6.4 Association between PCV2 viral load in OF and PCV2 viral load in serum samples

In the present study, two of the main objectives were to investigate if a correlation existed between OF and serum (all) (objective 1) and between OF and serum (chewers) (objective 2).

No strong correlation coefficient was obtained, neither between PCV2 viral load in OF and serum (all) nor between PCV2 load in OF and serum (chewers). Poor correlation coefficients were obtained, both when calculating Pearson's and Spearman's correlation coefficients. The poor correlation coefficients obtained in the present study are in accordance with the results from a previous study, where a low correlation coefficient of 0.39 (ρ) from pigs 13 weeks of age, as well as no correlation from pigs 19 weeks of age were obtained (Nielsen, 2017). However, higher correlation coefficients between PCV2 viral load in OF/nasal swabs and PCV2 viral load in serum have also been obtained (Grau-Roma *et al.*, 2009; Kim, 2010; Nielsen, 2017). On an individual animal level, a moderate correlation coefficient of 0.66 (r) between PCV2 viral load in serum and PCV2 viral load in nasal swabs has been reported (Grau-Roma et al., 2009). Comparable correlation coefficients of 0.69 (ρ) and 0.78 (r) have been reported between OF and serum pools containing serum from four to five pigs (Kim, 2010; Nielsen, 2017). In the present study, the correlation coefficients were calculated based on positive samples, whereas the correlation coefficients calculated in previous studies also included negative samples. Nevertheless, Kim (2010) reported a Pearson's correlation coefficient, even though it is highly sensitive to outliers. This might explain the high correlation coefficient obtained in the study.

As previously mentioned, Nielsen (2017) suggested that when a higher proportion of pigs are blood sampled in each pen, the higher the correlation coefficient, seeing as the proportion of pigs contributing to the serum samples approaches the proportion of pigs contributing to the OF samples. However, this was not supported in the present study, as poor correlation coefficients were obtained between OF and serum pools, even though serum (chewers) contained the exact same pigs that contributed to the OF sample.

Instead, it could be speculated that differences in how the OF and serum pools are obtained (fixed amount of serum from each pig in serum pools vs. unknown amount of OF from each pig in OF sample) could reduce the chances of obtaining similar results from the sample types, thus affecting the correlation. It may be a possibility that the OF sample results are influenced by factors such as the following: The duration each individual pig chews on the rope, the amount of oral fluid deposited on the rope, as well as changes in the absorptive capability of the rope, as the rope becomes increasingly wet. This may cause the OF results to be affected, to a greater extent, by infection dynamics such as variations in PCV2 viral load in individual pigs and differences in within-pen

prevalence, further reducing the likelihood of obtaining similar results from the two sample types. Results from the individual serum samples revealed both a difference in within-pen prevalence, and a great variation in individual PCV2 viral loads within and between pens, which makes the aforementioned suggestion more likely. However, it is worth mentioning that within-pen prevalences in the present study were calculated from only seven pens.

Based on the results from the present study, it seems that it is not possible to determine the PCV2 viral load in serum from the PCV2 viral load in OF. Since a broader knowledge of the PCV2 viral load in serum in relation to clinical signs exists, it might be more reliable to use qPCR on serum pools for PCV2 quantification when interventions such as vaccinations are considered. To enhance the diagnostic quality of OF samples, further investigations are needed to determine at which PCV2 viral load the infection might have a clinical effect.

6.5 Association between PCV2 viral load in serum pool from all the pigs in the pen and PCV2 viral load in faeces

Another objective was to investigate whether a correlation between the PCV2 viral load in faeces from sock samples and PCV2 viral load in serum (all) existed. To the authors' best knowledge, no studies have previously been investigating this. However, since sock samples have been shown to offer a reliable diagnostic method for examination of both presence and excretion levels of bacterial pathogens involved in intestinal diseases (Pedersen et al., 2015) and PCV2 can be detected and quantified in individual faecal samples (Segalés et al., 2005; Grau-Roma et al., 2009; Patterson et al., 2011), it seemed reasonable to assume that sock samples could be used as an alternative method for determination of PCV2 viral load on a group level.

In the present study, all collected sock samples were positive for PCV2, which indicates that qPCR on faeces from sock samples can be used for detection of PCV2. However, the correlations between the PCV2 viral load in serum (all) and faeces were contradictory, since a positive (although not significant) and a negative correlation coefficient were obtained for Age-group 1 and Age-group 2, respectively. The negative correlation coefficient obtained in Age-group 2 was especially unexpected, since a high positive correlation between rectal swabs and serum samples on an individual animal level have previously been shown (Grau-Roma et al., 2009). Although different from the correlation coefficient obtained in Age-group 2, the low but non-significant correlation found in Age-group 1 is comparable to the results obtained in a previous Danish Veterinary Master's thesis (Leth, 2010). In a Danish finisher herd, a positive but not significant correlation of 0.31 between PCV2 viral load in

floor samples and serum pools was obtained based on samples from 50 pens across different age groups.

The poor correlations obtained in the present study might be due to the potential constraint that only pigs that have recently been defecating on the slatted floor area are represented in the sock sample. This might decrease the likelihood of obtaining similar results from the two samples types, thus affecting the correlation.

The unexpected negative correlation obtained in Age-group 2 seemed primarily to be because of four pens showing a similar low load in sock samples, while showing an increasing level in matched serum pools. This might be a coincidence or because the pigs with a high PCV2 viral load in serum were anorectic, thereby contributing with less faecal matter to the slatted floor area. However, if the pigs were anorectic because of general depression, it would be expected that these pigs would not be interested in chewing the rope. However, from the individual samples, most of the pigs with the highest viral loads in serum did chew the rope, and might therefore also be expected to eat and defecate.

Whatever the case, based on the results from the present study, it seems that it is not possible to determine the PCV2 viral load in serum from the PCV2 viral load in faces from sock samples. If sock samples should be implemented as a diagnostic method for quantification of PCV2 by qPCR, supplemental studies should be conducted, to determine both sensitivity and specificity of sock samples regarding PCV2, and at which load the infection might be suspected to have a clinical effect.

6.6 PCV2 viral load in serum pools and individual serum samples

One of the objectives was to assess the impact of PCV2 viral load in individual pigs on a pooled serum sample. Due to economic reasons only seven pens were chosen for qPCR analysis on serum from individual pigs in the pen.

Overall, no statistically significant difference in the PCV2 viral load in serum (all) and the PCV2 viral load in the matched serum (chewers) were observed. This could prompt one to think that the PCV2 viral load in serum from the pigs chewing the rope is representative for the average PCV2 viral load in the pen. However, the samples from individual pigs revealed that four out of seven pens contained only one or two pigs with a PCV2 viral load as high as the matched serum (all). Furthermore, the arithmetic mean, calculated from individual pigs' PCV2 viral load, was relatively close to the PCV2 viral load in serum (all), which was ≥ 1 $\log_{10}$ higher than the median PCV2 viral load in five out of the seven pens investigated. This indicates that pigs with a high viral load might have a dominant effect on the pool result. These findings are supported by Nielsen (2017), who, from

theoretical serum pools of five pigs, found that one pig could cause an apparently high pool viral load. However, it should be emphasized that the results obtained in the present study were based on only seven pens with a larger pool size than normally used. Therefore, it would be relevant to investigate whether the results are true for 'real' serum pools of five pigs.

Instead of determining the median PCV2 load in the pen, it might be of greater relevance to find pigs with a viral load above 7 $\log_{10}$ PCV2 copies/ml, as high PCV2 viral loads in individual pigs have been associated with disease (Brunborg, Moldal and Jonassen, 2004; Segalés et al., 2005; Grau-Roma et al., 2009). For this purpose, a suggested pool cut-off value of 6.7 $\log_{10}$ or 6.0 $\log_{10}$ PCV2 copies/ml may be used for pools containing serum from five and ten pigs, respectively (Cortey et al., 2011; Nielsen, 2017).

It is not known whether the proposed cut-off value would have applied to the serum pools used in this study, as they, in most cases, contained serum from more than ten pigs. It was not possible nor did it from the perspective of a veterinary practitioner seem relevant to propose a cut-off value for the large pool sizes used in this study, as serum pools containing more than five pigs are rarely investigated.

7 Conclusion

The objectives of the study were to investigate if a correlation existed between PCV2 viral load in 1) OF and serum from all the pigs in the pen, 2) OF and serum from pigs chewing the rope during OF collection, 3) OF and faeces from sock samples and to assess the impact of individual pigs' PCV2 viral load on a pooled serum sample. The results revealed that, neither a good agreement nor a strong correlation between PCV2 viral load in OF and serum (all) (Age-group 1: r = 0.5, p = 0.045 Age-group 2: r = 0.16, p = 0.64) or between PCV2 viral load in OF and serum (chewers) exists (Age-group 1: r = 0.51, p = 0.04, Age-group 2: r = 0.12, p =0.55). Differences in how the OF and serum pools are obtained, which could make OF more likely to be affected by the variation within and between pens, might explain the poor correlations.

Poor correlations were also obtained between PCV2 viral load in serum (all) and faeces from sock samples (Age-group 1: r = 0.35, p = 0.17 and Age-group 2: r = -0.51, p=0.04). The poor correlations obtained may be due to the potential constraint that only pigs that had recently been defecating on the slatted floor area were represented in the sock samples, whereas the serum pool contained all the pigs in the pen.

Whatever the reason for the obtained correlations, these results suggest that the PCV2 results in OF and faeces from sock samples cannot be used to determine the PCV2 viral load in serum in finisher pigs. If Danish veterinary practitioners interpret the PCV2 viral load in OF and faeces from sock samples using the same reference PCV2 viral load used when interpreting serum results, a risk of drawing misleading conclusions is present.

Therefore, it may be more reliable to use serum pools for quantification of PCV2, since a broader knowledge of the PCV2 viral load in serum in relation to clinical signs currently exists. OF may instead be used for monitoring the infection dynamics in the finisher herds.

From the individual serum samples, it seemed that pigs with a high PCV2 viral load could potentially dominate the pool result, making the pool result non-representative for the average PCV2 viral load in the pens. Instead of trying to find the average PCV2 viral load, serum pools may be used for detection of individual pigs with an apparent high PCV2 viral load.

8 Perspectivation

The present study indicated that the PCV2 viral load in OF could not be used to determine the PCV2 viral load in serum in Danish finisher pigs. However, since OF collection is of lower stress to the animals, are of lower cost to the farmer and because PCV2 infection might be detected at an earlier time and at lower prevalences, it may be of interest to enhance the diagnostic quality of OF. It would therefore be relevant to further investigate at which PCV2 viral load the infection might have a clinical effect. The abovementioned also applies to sock samples, and it would furthermore be relevant to estimate the sensitivity and specificity of sock samples regarding PCV2.

Due to the cross-sectional nature of the study, it was not possible to assess whether the infection dynamics were the main reason for the differences in the PCV2 results between the samples as well as the poor correlations. To see if this could be the case, it would be interesting to investigate both serum pools and OF samples in a longitudinal study.

From the individual samples, it was observed that only four out of seven pens contained pigs with a PCV2 viral load as high as the serum (all), which could indicate that one pig with a high viral load could dominate the pool result. Therefore, it would be interesting to see if this applies to ‘real’ pools of five pigs as well. Furthermore, it could be interesting to investigate if one pig could have a similar dominant effect on the PCV2 viral load in OF and sock samples.

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10 Appendix

A. Schema for registrations of chewing and non- chewing pigs

Besætning nummer:
Dato for prøveudtagning:
Antal grise i stien:
Alder i stien:

Sti nummer	Gris nr	Bider i rebet	Bider ikke i rebet	Kommentarer
	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	11			
	12			
	13			
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	25			
	26			
	27			
	28			
	29			
	30			

B. qPCR results

B.1 Pooled samples

Age-group 1	Log10 PCV2 copies pr. ml serum (all)	Log10 PCV2 copies pr. ml serum (chewers)	Log10 PCV2 copies pr. ml oral fluid	Log10 PCV2 copies pr. g feces
Pen 1	4,58	4,53	5,74	5,2
Pen 2	5,59	5,43	6,53	5,45
Pen 3	4,68	4,68	5,88	4,9
Pen 4	6,35	6,26	6,84	5,61
Pen 5	5,25	5,32	6,49	5,56
Pen 6	4,91	5,29	6,38	5,69
Pen 7	4,46	4,68	6,58	5,55
Pen 8	4,49	4,79	7,12	5,75
Pen 9	5,74	5,79	6,7	5,5
Pen 10	5,52	5,46	8,01	7,25
Pen 11	5,19	5,41	6,72	5,54
Pen 12	6,06	6,33	7,2	5,97
Pen 13	5,7	5,65	7,08	6,39
Pen 14	6,42	5,17	7,03	5,76
Pen 15	5,55	5,59	6,55	5,27
Pen 16	5,82	5,84	7,55	6,55
Pen 17	5,74	5,82	6,84	5,86

Age-group 2	Log10 PCV2 copies pr. ml serum (all)	Log10 PCV2 copies pr. ml serum (chewers)	Log10 PCV2 copies pr. ml oral fluid	Log10 PCV2 copies pr. g feces
Pen 1	4,35	4,16	5,94	5,12
Pen 2	5,7	5,46	5,74	4,69
Pen 3	6,7	6,2	5,87	4,81
Pen 4	4,58	4,58	5,75	4,94
Pen 5	5,01	5,08	6,42	5,05
Pen 6	4,83	4,72	5,45	5,13
Pen 7	5,42	5,55	6,5	5,43
Pen 8	4,63	5,01	5,88	5,16
Pen 9	4,78	4,67	6,18	5,52
Pen 10	4,96	5,05	5,96	5,21
Pen 11	5,1	4,63	6,58	4,94
Pen 12	5,47	5,23	5,77	5,29
Pen 13	5,28	5,01	7,68	5,41
Pen 14	5,38	5,44	6,41	5,29
Pen 15	5,39	5,46	5,74	4,93
Pen 16	6,09	6,27	6,28	4,69
Pen 17	6,45	6,45	6,57	4,79

B.2 Individual serum samples Age-group 1

Pen 3, Age-group 1		
Number	**PCV2 copies/ml serum**	**Log10 PCV2 copies/ml serum**
17-12673-1	0	0
17-12673-2	373000	5,57
17-12673-3	328000	5,52
17-12673-4	380000	5,58
17-12673-5	6020	3,79
17-12673-6	5070	3,7
17-12673-7	5370	3,73
17-12673-8	0	0
17-12673-9	0	0
17-12673-10	460000	5,66
17-12673-11	29500	4,47
17-12673-12	63300	4,8
17-12673-13	1820	3,26
17-12673-14	0	0
17-12673-15	5570	3,75

Pen 10, Age-group 1		
Number	**PCV2 copies/ml serum**	**Log10 PCV2 copies/ml serum**
17-12680-1	50000	4,7
17-12680-2	103000	5,12
17-12680-3	2800	3,45
17-12680-4	177000	5,25
17-12680-5	196000	5,29
17-12680-6	2770000	6,44
17-12680-7	167000	5,22
17-12680-8	7330	3,87
17-12680-9	0	2
17-12680-10	55000	4,74
17-12680-11	16300	4,21
17-12680-12	563000	5,75
17-12680-13	16600	4,22
17-12680-14	31100	4,49
17-12680-15	38700	4,59
17-12680-16	1470000	6,17
17-12680-17	68700	4,84
17-12680-18	900000	5,95
17-12680-19	493000	5,69
17-12680-20	205000	5,31
17-12680-21	873000	5,94

Pen 14, Age-group 1		
Number	**PCV2 copies/ml serum**	**Log10 PCV2 copies/ml serum**
17-12684-1	57000	4,76
17-12684-2	553000	5,74
17-12684-3	39700000	7,6
17-12684-4	228000	5,36
17-12684-5	10200	4,01
17-12684-6	233000	5,37
17-12684-7	74300	4,87
17-12684-8	35300	4,55
17-12684-9	730000	5,86
17-12684-10	2680	3,43
17-12684-11	166000	5,22
17-12684-12	2210	3,34
17-12684-13	1710	3,23
17-12684-14	11000	4,04
17-12684-15	276000	5,44
17-12684-16	123000	5,09
17-12684-17	743000	5,87

B.3 From individual serum samples Age-group 2

Pen 1, Age-group 2		
Number	**PCV2 copies/ml serum**	**Log10 PCV2 copies/ml serum**
17-12688-1	860	2,93
17-12688-2	3930	3,59
17-12688-3	2500	3,4
17-12688-4	6070	3,78
17-12688-5	0	0
17-12688-6	20300	4,31
17-12688-7	4300	3,63
17-12688-8	0	0
17-12688-9	71700	4,86
17-12688-10	17900	4,25
17-12688-11	0	0
17-12688-12	0	0
17-12688-13	0	0
17-12688-14	0	0
17-12688-15	1950000	6,29
17-12688-16	427000	5,63

Pen 2, Age-group 2		
Number	**PCV2 copies/ml serum**	**Log10 PCV2 copies/ml serum**
17-12689-1	0	0
17-12689-2	12100	4,08
17-12689-3	2420000	6,38
17-12689-4	12200	4,09
17-12689-5	523000	5,72
17-12689-6	7270	3,86
17-12689-7	0	0
17-12689-8	0	0
17-12689-9	0	0
17-12689-10	3060	3,49
17-12689-11	107000	5,03
17-12689-12	11700	4,07
17-12689-13	0	0
17-12689-14	2290	3,36
17-12689-15	4230	3,63
17-12689-16	7970	3,9

Pen 11, Age-group 2		
Number	**PCV2 copies/ml serum**	**Log10 PCV2 copies/ml serum**
17-12698-1	6230	3,79
17-12698-2	180000	5,26
17-12698-3	2540	3,4
17-12698-4	19700	4,29
17-12698-5	2040	3,31
17-12698-6	6170	3,79
17-12698-7	8170	3,91
17-12698-8	2750	3,44
17-12698-9	0	0
17-12698-10	19700	4,29
17-12698-11	2090	3,32
17-12698-12	2370	3,37
17-12698-13	1260	3,1
17-12698-14	0	0
17-12698-15	0	0
17-12698-16	36700	4,56

Pen 17, Age-group 2		
Number	**PCV2 copies/ml serum**	**Log10 PCV2 copies/ml serum**
17-12704-1	0	0
17-12704-2	104000	5,02
17-12704-3	0	0
17-12704-4	0	0
17-12704-5	0	0
17-12704-6	0	0
17-12704-7	259000	5,41
17-12704-8	2380	3,38
17-12704-9	70000	4,85
17-12704-10	52700	4,72
17-12704-11	2420000	6,38
17-12704-12	77000	4,89
17-12704-13	1670	3,22
17-12704-14	0	0
17-12704-15	630000	5,8
17-12704-16	1520000	6,18