Technical University of Denmark DTU

MASTER'S THESIS

Porcine reproductive and respiratory syndrome virus – dynamics, persistence and genetic characterization in three Danish swine herds following blitz vaccination

Porcint reproduktions- og respirations syndrom virus – dynamik, persistens og genetisk karakterisering i tre danske svinebesætninger efter massevaccination

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Preface

This study was conducted at the Section for Virology, National Veterinary Institute (NVI), Technical University of Denmark (DTU), from 1st of September 2016 to 15th of January 2017. The study was executed as the completion of the master's programme in veterinary medicine at the University of Copenhagen by veterinary students Josefine Meyer Jørgensen and Sarah Nielsen.

The main supervisor was Lars Erik Larsen, professor at Section for Virology, NVI, DTU. Contributing supervisors were Jens Peter Nielsen, professor at Section for Production and Health, University of Copenhagen, and Lise Kirstine Kvisgaard, ph.d. and post.doc. at Section for Virology, NVI, DTU. External supervisors were: Charlotte Sonne Kristensen, team manager health, DVM and ph.d. at SEGES Pig Research Centre, Anders Elvstrøm, DVM, certified in swine herd health at Danvet, and Jakob Bagger, DVM, certified in swine herd health at Boehringer Ingelheim. All supervisors are sincerely thanked for their guidance and help.

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Abstract

Introduction: Porcine reproductive and respiratory syndrome (PRRS) is a highly infective and worldwide economically important disease caused by PRRS virus (PRRSV). Constant viral genetic change together with a long-term carrier state and debatable immunity frequently confound attempts to eradicate PRRSV in swine herds. Furthermore, genetically different variants of PRRSV often co-exist within the same herd. The Danish swine herds positive of PRRSV have often been through an extensive elimination programme (vaccination, herd sanitization), yet for some herds, it is impossible to get control of the PRRSV.

Aims: To investigate the dynamics, persistence and genetic characteristics of PRRSV in presumed stabilized and sanitized swine herds. We explored why these herds are PRRSV positive and in which sections the infection occurs. In addition, we wanted to compare different diagnostic approaches for detecting PRRSV.

Methods: This cross-sectional study examined PRRSV status in different age groups (pigs at age 0, 4, 8 and 12 weeks). It consisted of two parts: A management related observational study (questionnaire, clinical registrations), and a virus detection and genetic characterization study with collection of blood serum samples, tonsil swabs, air samples, and placenta-umbilical cord serum (PUCS) samples. Samples were screened for PRRSV (real time RT-PCR), and some were further chosen for sequencing to explore genetic variations (Sanger and Next-Generation Sequencing). A two-sided p-value lower than 0.05 was considered statistically significant.

Results: None of the herds followed McRebel completely. Based on clinical registrations, all case herds had low occurrence of symptoms. All air samples and PUCS samples were PRRSV negative. Positive blood serum samples were seen in herd 1 (12 weeks), herd 2 (8 and 12 weeks), and herd 3 (4, 8 and 12 weeks). The exact same pooled blood serum and tonsil swab samples (consisting of the exact same single samples/pigs) from pigs at 12 weeks of age were found PPRSV positive. However, Ct-values from blood serum samples were significantly lower ($p = 0.003$) than from tonsil swabs. Sanger sequencing (ORF5) showed strong resemblance to Ingelvac PRRS® MLV (ranging between 98.8-99.5%). No quasispecies were found using NGS Ion Torrent.

Conclusions: None of the three herds followed McRebel strictly and were all PRRSV positive. Yet, occurrence of clinical symptoms was low (cannot indicate PRRSV status). None of the piglets (0 weeks) tested PPRSV positive. Blood serum seemed more sensitive than tonsil swabs for detecting PRRSV. No quasispecies were found. The sequences from the circulating virus in the three herds had high similarity to Ingelvac PRRS® MLV.

Resumé

Introduktion: Porcint reproduktions- og respirations syndrom (PRRS) er en smitsom og globalt økonomisk vigtig sygdom forårsaget af PRRS virus (PRRSV). Konstant ændring i viralt genom samt langvarig bærertilstand og tvivlsom immunitet gør det ofte svært at udrydde PRRSV fra besætninger. Forskellige genetiske varianter af virusset eksisterer ofte indenfor samme besætning. Danske PRRSV positive svinebesætninger har ofte gennemgået et omfattende eliminationsprogram (vaccination, sanering). Til trods herfor er det ofte svært at få kontrol over PRRSV.

Formål: At undersøge dynamik, persistens og genetiske karakteristika af PRRSV i formodede stabile og sanerede svinebesætninger. Vi undersøgte hvorfor disse besætninger var PRRSV positive samt i hvilke sektioner, infektionen fandt sted. Desuden ville vi sammenligne forskellige måder, hvorpå PRRSV kan diagnosticeres.

Metoder: Dette tværsnitsstudie undersøgte PRRSV status i forskellige aldersgrupper (0, 4, 8 og 12 ugers grise), med fokus på to områder: Et management-relateret observationelt studie (spørgeskema, kliniske registreringer), samt et viralt detektions- og genetisk karakteriseringsstudie med udtagning af blodprøver, tonsil svaber, luftprøver og placenta-navlesnor serum (PUCS) prøver. Prøverne blev screenet for PRRSV (real time RT-PCR), og nogle prøver blev udvalgt til sekventering for at undersøge genetisk variation (Sanger og Next-Generation Sequencing). En to-sidet p-værdi under 0.05 blev tolket som statistisk signifikant.

Resultater: Ingen af besætningerne fulgte McRebel fuldstændigt. Baseret på kliniske registreringer havde alle besætningerne lav forekomst af symptomer. Alle luftprøver og PUCS prøver fandtes PRRSV negative. Positive blodprøver fandtes i besætning 1 (12 ugers), besætning 2 (8 og 12 ugers), samt besætning 3 (4, 8 og 12 ugers). De nøjagtigt samme poolede blod- og tonsilprøver (indeholdende de præcist samme enkeltprøver/grise) fra 12 ugers grise fandtes PRRSV positive. Dog var Ct-værdierne fra blodprøverne signifikant lavere ($p = 0.003$) end fra tonsilsvaberne. Sanger sekventering (ORF5) viste stor lighed med Ingelvac PRRS® MLV (mellem 98.8-99.5%). Ingen quasispecies blev fundet med NGS Ion Torrent.

Konklusioner: Ingen af de tre besætninger fuldte McRebel fukdstænding, og alle fandtes PRRSV positive. Tilstedeværelsen af kliniske symptomer var lav (kan ikke indikere PRRSV status). Ingen af pattegrisene (0 ugers) blev testet PRRSV positive. Blodserum virker til at være mere sensitiv end tonsilsvaber ift. at detektere PRRSV. Ingen quasispecies blev fundet. Sekvenserne fra cirkulerende virus i de tre besætninger udviste stor lighed med Ingelvac PRRS® MLV.

Abbreviations

ADD: Average daily dose

AI/AO: All in/all out

DNA: Deoxyribonucleic acid

dNTP: Nucleoside triphosphates containing deoxyribose

dsDNA: Double-stranded deoxyribonucleic acid

dsRNA: Double-stranded ribonucleic acid

dpv: Days post vaccination

ELISA: Enzyme-linked immunosorbent assay

GIS: Geographic information system

IMPA: Immunoperoxidase monolayer assay

IFN: Interferon

IPT: Immunoperoxidase test

kb: Kilobases

McRebel: Management changes to reduce exposure to bacteria and eliminate losses

MLV: Modified live vaccine

NGS: Next-Generation Sequencing

nm: Nano meters

NSP: Non-structural protein

ORF: Open reading frame

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PCV2: Porcine circovirus 2

PI: Post infection

PRRS: Porcine reproductive and respiratory syndrome

PRRSV: Porcine reproductive and respiratory syndrome virus

PUCS: Placenta-umbilical cord serum

Real time RT-PCR: Real time reverse transcriptase polymerase chain reaction

RNA: Ribonucleic acid

RT: Room temperature

SPF: Specific pathogen free

$TCID_{50}$: Tissue culture infective dose

VSP: Videncenter for Svineproduktion

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a worldwide economically important swine disease caused by PRRS virus (PRRSV). PRRS is characterized by reproductive failure (multiple abortions, stillbirths, weak born piglets) in sows, and reduced growth rate and feed efficiency as well as pneumonia and impaired animal welfare in the growing pigs (Christianson, W. T. et al., 1993; Collins, J. E. et al., 1992). Respiratory symptoms may occur at any age but are especially seen in weaners and finishers. Furthermore, infected pigs are more susceptible to secondary infections presumably due to the affected immune system. Such infections could be pneumonia caused by *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae*, which in PRRSV positive pigs are more difficult to control. In addition, encephalitis and arthritis caused by *Streptococcus suis,* worsening of impetigo caused by *Staphylococcus hyicus*, and worsening of diarrhea are often seen as well (Vestergaard, K. et al., 2007).

PRRSV was first recognized in the United States in 1987 and appeared in Europe in 1990 (Zimmerman, J., 2003). It was later suggested that the PRRSV originated from wild boars and was spread to North America through the import of animals in 1912 (Plagemann, P. G. W., 2003). It was diagnosed in 1992 (type 1) in Denmark and is now widely disseminated throughout the country. An introduction of a PRRSV modified live vaccine (MLV) came in 1996, which led to a new strain of PRRSV (type 2) among Danish swine herds. Clinical symptoms of PRRS vary from herd to herd regardless of viral strain (Kristensen, C. S. et al., 2014a). It is estimated that 50% of the herds in Denmark are seropositive for PRRSV type 1, type 2 or both (Kvisgaard, L. K. et al., 2013). PRRSV negatively impacts productivity in Denmark by reducing production up to 30 weeks after introduction of PRRSV leading to an estimated loss of 330 DKK/sow/year (Kristensen, C. S. et al., 2013a). This has led to greater focus on viral control and/or elimination.

For some herds, it has been impossible to eliminate the PRRSV even though farmers in cooperation with veterinarians have executed extensive vaccination programmes and implemented management related tools such as the McRebel guidelines (Kristensen, C. S. et al., 2014a; McCaw, M, 2003).

This master's thesis wants to investigate and give a more comprehensive and contemporary understanding of the dynamics, persistence and genetic characteristics of PRRSV in 3 Danish swine herds, which have been blitz vaccinated (a vaccination strategy, where pigs in certain age groups all

have been vaccinated twice with three weeks apart). In addition, we want to examine and compare different tools for diagnosing and detecting PRRSV. Hopefully, this can lead to suggestions on how management can be optimized and/or PRRSV (re)occurrence prevented.

The following null-hypotheses were tested:

- *H_0 number 1: There is no significant difference in PRRSV occurrence when comparing real time RT-PCR results from PUCS and tonsil swabs from 0 weeks old pigs.*
- *H_0 number 2: There is no significant difference in PRRSV occurrence when comparing real time RT-PCR results from blood serum and tonsil swabs from 12 weeks old pigs.*
- *H_0 number 3: There is no significant difference between virus load (Ct-values) in the different age groups.*
- *H_0 number 4: There is no significant difference between PRRSV quasispecies and the age of the pigs.*
- *H_0 number 5: There is no significant difference between signs of clinical illness and PRRSV status in blood serum.*

Theoretical background

1 History and virology

1.1 Taxonomy, structure and genomic orientation

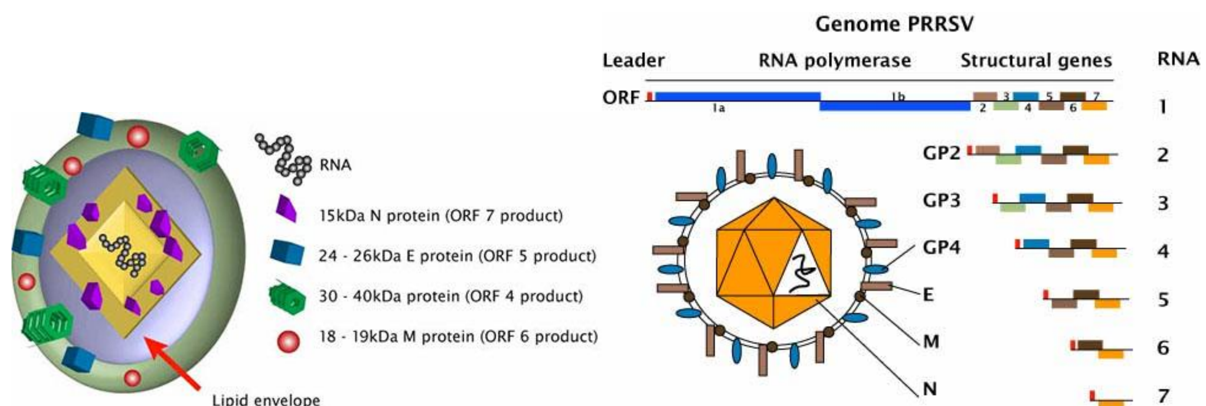
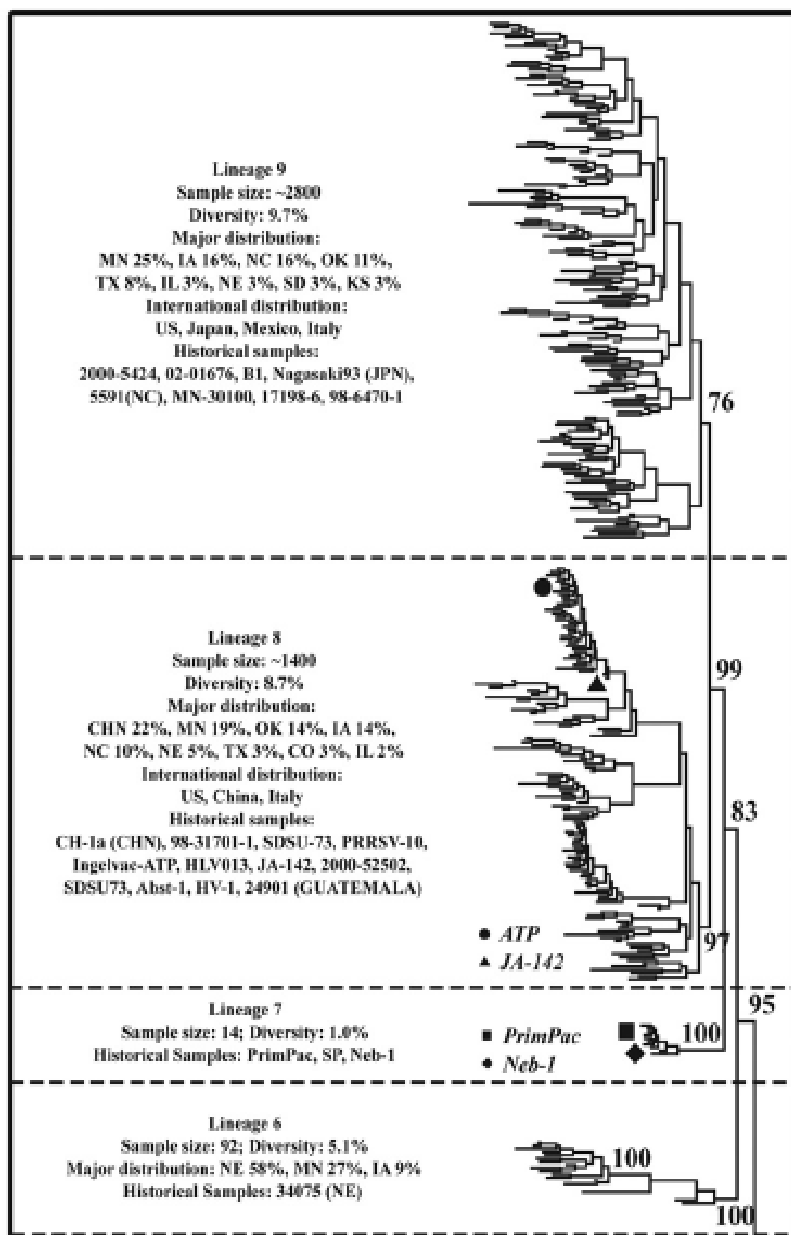


Figure 1: Virus structure (left) and genome (right). (Source: Genome PRRSV, 2016).

PRRSV is a small, enveloped, positive sense, single-stranded RNA virus. It belongs to the family *Arteriviridae*, which is a member of the *Nidovirales* order (Benfield, D. A. et al., 1992; Meulenberg,

J. J. M. et. al., 1993; Cavanagh, D., 1997). The morphology of the virus is pleomorphic with an oval/spherical shape and a size ranging from 50-65 nm. The core is hollow, about 40 nm in size, and has an even external surface containing envelope protein complexes (Spilman, M. S. et al., 2009). The virus genome is surrounded by a nucleocapsid, which is again surrounded by a lipid membrane containing structural protein complexes (Spilman, M. S. et al., 2009; Benfield, D. A. et al., 1992; Wensvoort, G. et al., 1991). The lipid membrane affects the virus’ ability to survive outside a host cell and makes it heat labile in the range 37-56 ℃ but reasonably stable from −70 to +4 ℃ (Benfield, D. A. et al., 1992).



PRRSV (see Figure 1) has a 15 kb long genome with a methyl-capped 5’-end, a polyadenylated 3’-end, and 10 open reading frames (ORF) encoding virus specific proteins (Meulenberg, J. J. M. et al., 1993; Conzelmann, K. et al., 1993; Wu, W. et al., 2001; Kimman, T. G. et al., 2009; Firth, A. E. et al., 2011). ORF1 (parts a and b) constitutes approximately 75% of the genome and codes for 2 non-structural proteins (NSPs). PRRSV encodes several structural proteins: GP2, E, GP4, GP5, M, N-protein, and ORF5a protein (Firth, A. E. et al., 2011; Johnson, C. R. et al., 2011). ORF2 encodes GP2, which together with GP4 is suspected to be involved in the uncoating of the virus (Das, P. B.

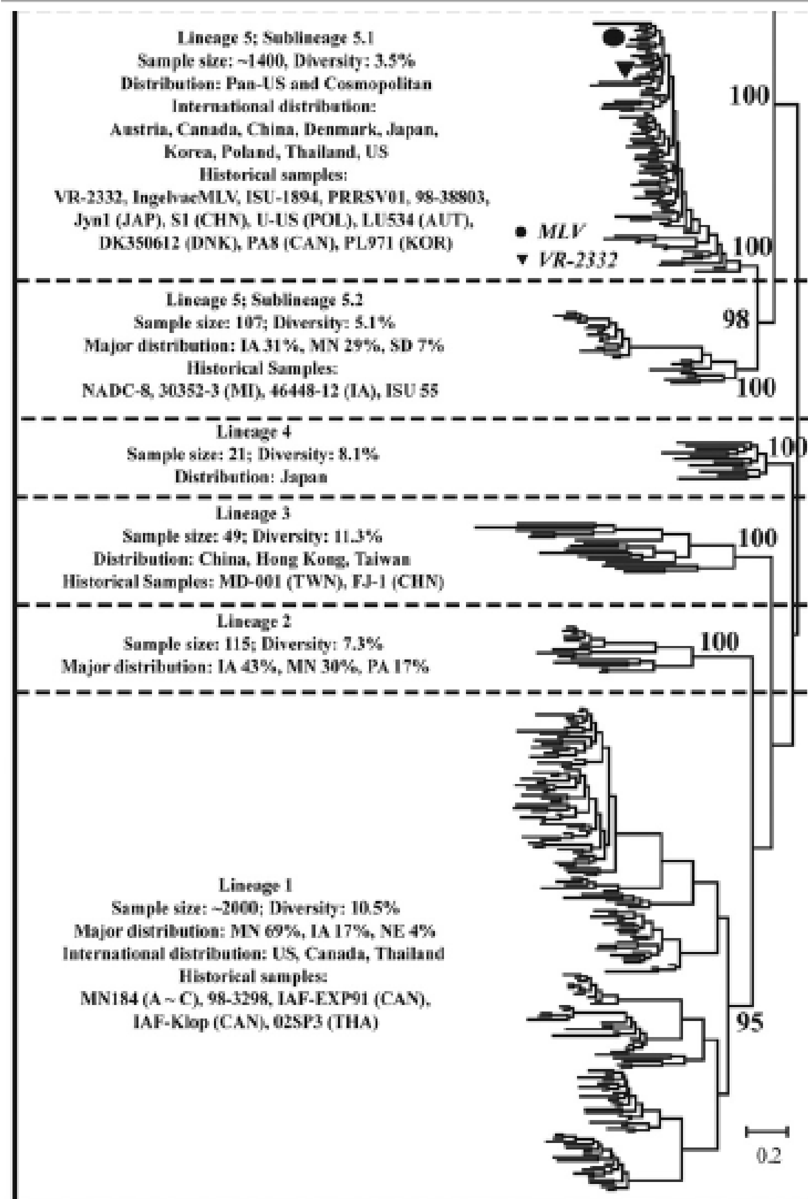


Figure 2. Phylogeny of PRRSV type 2. Lineage 5 contains the Danish type 2 isolates (sublineage 5.1). Other isolates in this lineage are from Austria, Canada, China, Japan, Korea, Poland, Thailand, and the United States. This sublineage emerged in 1986-89 (Shi, M. et al., 2010a). (Source: Phylogenetic tree type 2, 2016).

et al., 2010). ORF 3 to 5 encode membrane glycoproteins (GP3-5), ORF6 encodes a non-glycosylated membrane protein (M), and ORF7 encodes a nucleocapsid protein (N) (Firth, A. E. et al., 2011; Johnson, C. R. et al., 2011; Wu, W. et al., 2001). In PRRSV type 2, the GP5 contains a decoy epitope, which might be able to distract the humoral immune system and thereby delaying the antibody response (Ostrowski, M. et al., 2002).

1.2 Types, genetic diversity and phylogeny

The focus of this thesis is on PRRSV type 2, but both type 1 and type 2 will be presented shortly. Though the pigs infected with PRRSV present with similar clinical symptoms, the antigenic structure and genetic organization of PRRSV vary greatly (Shi, M. et al., 2010a). There are two different PRRSV genotypes: Type 1 (European type), and type 2 (North Ameri-can type). Originally, it was believed that PRRSV type 2 contained more genetic diversity than type 1 (Forsberg, R. et al., 2002; Martınez-Lobo, F. J. et al., 2011). This theory was dispelled after a comprehensive study of type 1 documenting that the Euro-

pean type was actually more diverse 2002; Stadejek, T. etthan the North American type (Meng, X. J. et al., 1995; Kapur, V. et al., 1996; Suarez, P. et al., 1996; Stadejek, T. et al., al., 2006; Stadejek, T. et al., 2008). The first two isolates discovered were a type 1 called Lelystad virus (LV) and a type 2 called American Type Culture Collection VR-2332 (ATCC VR-2332) (Wensvoort, G. et al., 1991; Collins, J. E. et al., 1992). Genomic analyses of the two virus types revealed significant differences between the European and the American types with only 60% nucleotide similarity and thereby concluding that two different genotypes of the PRRSV exist (Allende, R. et al., 1999).

Based on comparison of the ORF5 sequence from different PRRSV type 2 isolates, it has been demonstrated that PRRSV type 2 can be divided into 9 different lineages (see Figure 2). The North American isolates are the most diverse since they are found in 7 of 9 lineages (Shi, M. et al., 2010a). The Danish isolates are found in lineage 5 (sublineage 5.1) together with the Ingelvac PRRS® MLV vaccine (marked with a dot in Figure 2) and the ATCC VR-2332 (marked with a triangle in Figure 2) (Collins, J. E. et al., 1992; Stadejek, T. et al., 2013; Shi, M. et al., 2010a). The type 2 isolates found in Europe have until now been very similar (more than 98%) to the Ingelvac PRRS® MLV vaccine (Greiser-Wilke, I. et al., 2010; Stadejek, T. et al., 2013).

There are two main reasons for the production of different PRRSV quasispecies. RNA viruses have a higher occurrence of recombination and no proofreading system. These factors contribute to a high number of mutations (an average mutation rate of 1 mutation per 10.000 nucleotides) resulting in a large genetic and antigenic drift and development of quasispecies (Lauring, A. S. et al., 2010; Shi, M. et al., 2010b).

1.3 Pathogenesis and clinical manifestations

Infection with PRRSV can occur through several different routes. The most frequent route is the respiratory route, and the virus is capable of being airborne for long distances. Furthermore, it has also been found to be able to infect across the placenta and through semen (Rossow, K. D. et al., 1994; Christianson, W. T. et al., 1993; Bøtner, A. et al., 1994; Kristensen, C. S. et al., 2004; Yaeger, M. J. et al., 1993). When the PRRSV has infected a pig, it has a preference of targeting the alveolar macrophages in the lungs and the lymphoid tissue. These targets then become the primary replication site (Benfield, D. A. et al., 1992; Conzelmann, K. et al., 1993; Duan, X. et al., 1997; Suarez, P. et al., 2000). The virus enters the cell of the host through receptor-mediated endocytosis, and the vi-

rus is replicated in the cytoplasma (Duan, X. et al., 1998; Snijder, E. J. et al., 1998). Heparan sulfate facilitates first binding of PRRSV. This interaction is mediated by the M protein in cooperation with GP5 resulting in an increasing interaction with porcine sialoadhesin (PoSn). PoSn and CD163 receptor are essential for the attachment, internalization and uncoating of PRRSV (Kimman, T. G. et al., 2009).

Clinical manifestations in pigs vary remarkably according to the age and reproductive stage of the infected pig. Symptoms observed in sows and gilts are: Anorexia, lethargy, fever, and reproductive problems such as late time abortions, weak born piglets, mummified fetuses, and stillbirths (Christianson, W. T. et al., 1993; Bøtner, A. et al., 1994; Batista, L. et al., 2002). In growing pigs, infection with PRRSV can result in reduced growth rate, reduced feed efficiency, pneumonia, and general impaired animal welfare (Christianson, W. T. et al., 1993; Collins, J. E. et al., 1992).

1.4 Immunology

When the pig is infected by PRRSV and the alveolar macrophages are attacked, the immune response from the adaptive immune system is found to be very slow and faulty. The PRRSV appear to use multiple evasion techniques to evade both innate and acquired immune system by interfering with antigen presentation, antibody mediated enhancement, downregulated cell surface expression of viral proteins, and concealing neutralizing epitopes (Kimman, T. G. et al., 2009). The polyclonal activation of B-cells (producing IgG and IgM) takes place 7 to 9 days post infection (PI) (see Figure 3). At that time, antibodies directed predominantly against the N-protein can be detected through enzyme-linked immunosorbent assay (ELISA) and immunoperoxidase monolayer assay (IMPA). Four weeks PI, it is possible to detect interferon-γ (IFN-ɣ) producing T-cells as a sign of activation of the macrophages, which is an important step for the activation of the innate immune response. Upregulation of certain cytokines such as IL-10 can possibly contribute to the downregulation of important pro-inflammatory cytokines and Th-1 activity. Furthermore, natural killer cell activation is likely delayed resulting in a slow neutralizing antibody, lymphoproliferative, and IFN-γ response (Kimman, T. G. et al., 2009). It can take up to 3 months for the immunity to kick in, yet a reinfection with a different PRRSV quasispecies containing a different antigen structure (epitope) often cannot be prevented. Furthermore, the weak immune response may contribute to a long survival of PRRSV in infected pigs (Bautista, E. M. et al., 1999; Lopez, O. J. et al., 2004; Beura, L. K. et al.,

2010; Murtaugh, M. P. et al., 2011; Zuckermann, F. A. et al., 2007; Kimman, T. G. et al., 2009; Johnson, C. R. et al., 2004).

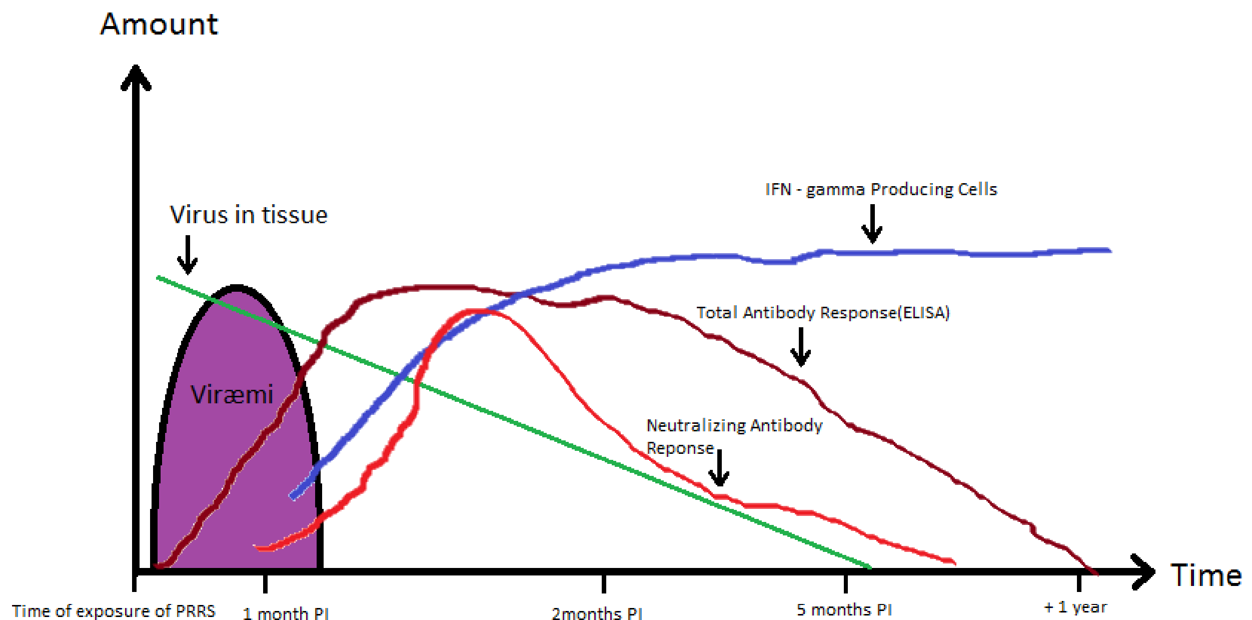


Figure 3. The immunological development during PRRSV infection in a pig. A high viral load is found in the early stage. The antibody response can be detected from 7 to 9 days PI and declines quickly during the first year (Source: Lopez, O. J. et al., 2004; Johnson, C. R. et al., 2004).

1.5 Viremia, excretion and persistent infection

The viremic period of a PRRSV infected pig has been investigated in experimentally infected animals, and researchers have found that viremia starts within 3 days PI and can last for up to 62 days post vaccination (dpv) (Yoon, I. et al., 1993; Kristensen, c. S. et al., 2016). Furthermore, the virus has even been detected through real time RT-PCR in up to 271 days PI (Rossow, K. D. et al., 1994; Rossow, K. D. et al., 1995; Bøtner, A. et al., 1994; Batista, L. et al., 2002; Duan, X. et al., 1997; Hermann, J. R., 2005; Prieto, C. et al., 2004; Lopez, O. J. et al., 2004; Wills, R. W. et al., 2003). The amount of virus excreted and the duration of excretion are dependent on the specific quasispecies of the virus isolate. However, it has been found that after the acute phase, the infected animals can carry the virus in their lymphoid tissue for a longer time period harboring a persistent infection that continuously sheds virus at low levels (Johnson, C. R. et al., 2004; Wills, R. W. et al., 1997a; Allende, R. et al., 2000). As described earlier, the PRRSV mainly replicates in the alveolar macrophages, which thereby distribute the virus to lymphoid tissues such as thymus, spleen, tonsils,

and lymph nodes. Additionally, it is also spread to the lungs, heart, aorta, kidneys, testes, salivary glands, gastrointestinal system, and brain (Christianson, W. T. et al., 1993; Rowland, R. R. R. et al., 2003).

It has been shown that PRRSV can be shed from the animals through nasal discharge, urine, oral fluids, feces, mammary glands, and semen. The nasal discharge, urine, oral fluids, feces, and semen have been found to shed virus 42-92 days PI, while mammary gland excretion has been found to take place up to 9 days after farrowing (Christopher-Hennings, J. et al., 1995; Wills, R. W. et al., 1997b; Yoon, K. et al., 1993; Wagstrom, E. A. et al., 2001).

2 PRRSV in a Danish perspective

2.1 Diagnostic tools and detection of PRRSV in Denmark

PRRSV is an endemic disease, and both type 1 and 2 are distributed all around the world (Shi, M. et al., 2010b). More than 50% of Danish swine herds are infected with type 1 and/or type 2 (Kvisgaard, L. K. et al., 2013). Moreover, around 78% of all sows are declared specific pathogen free (SPF). When herds are declared SPF, their PRRSV status must be registered as either positive or negative. If positive, the virus type must also be specified (SPF-sundhedsstyring, 2016). As a surveillance assay, blood samples detecting PRRSV are collected either once a year or once a month depending on the PRRSV status of the herd. PRRSV can be detected through either specific antibodies using blocking ELISA, Idexx ELISA or IPT (immunoperoxidase test), or viral RNA using real time RT-PCR. Through blocking ELISA, it is possible to detect antibodies as early as 8-14 days PI and up to two years PI. Through Idexx ELISA, it is possible to detect antibodies as early as 8-10 days PI and up to 193 days PI. With IPT, antibodies can be detected at the earliest 7-10 days PI and up to 6-10 months PI. If a herd is suspected of being PRRSV positive, the best way of detecting the virus is by making an ELISA and a real time RT-PCR. However, herds where virus is detected by real time RT-PCR are put on the Danish Veterinary and Food Administration's ("Fødevarestyrelsens") list over positive herds, and restrictions will be imposed regarding slaughtering of the pigs. The detection of PRRSV in a herd is therefore problematic in relation to export, since some countries will not receive meat from herds, where PRRSV has been found within the last year. When screening for PRRSV in a herd, samples are taken from a specific portion of pigs at different age groups. Viral detection is either carried out at the National Veterinary Institute (NVI), Technical

University of Denmark (DTU), or at SEGES Pig Research Centre, Kjellerup (Kristensen, C. S. et al., 2014a; Duinhof, T. F. et al., 2011; Bekendtgørelse om Porcin Reproduktions- og Respirationssygdom (PRRS) nr 314 af 26/04/94, 1994).

2.2 Incentives on herd level to become PRRSV negative

PRRSV’s impact on productivity in Denmark has previously been studied, and it was found that infection had a negative influence on productivity up to 30 weeks after introduction of the virus. In sows, PRRSV infection can result in reproductive failure with a lower farrowing rate, fewer total born piglets per litter, stillbirths, and a higher piglet mortality (see Figure 4). In growing pigs, PRRSV infection can result in lower daily gain, higher mortality, changes in meat percentage, and increased management workload because the staff has to spend more time caring for the pigs. By comparing productivity data from positive herds with data from negative herds, it was found that herds with PRRSV had a higher total piglet mortality and a higher weaner mortality (Kristensen, C. S. et al., 2013c).

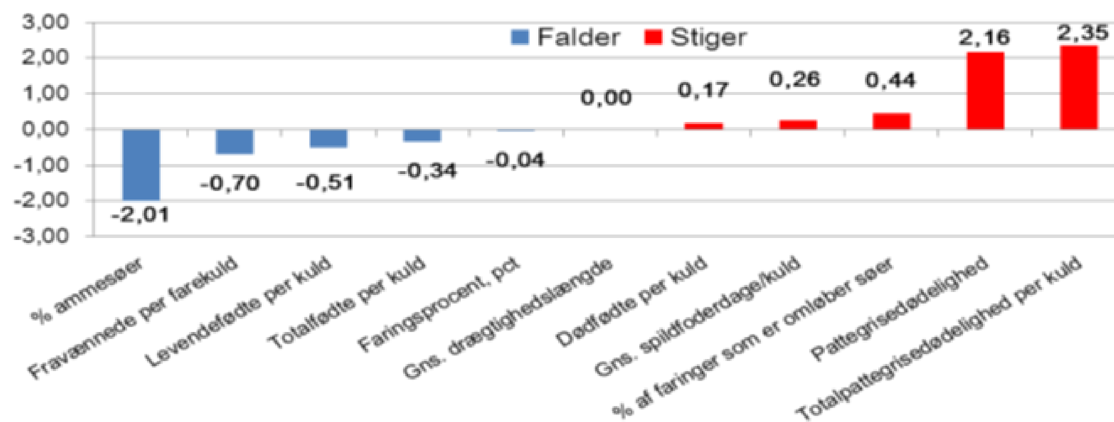


Figure 4. Changes regarding productivity in PRRSV positiv herds. (Kristensen, C. S. et al., 2013c).

No significant differences in the use of antibiotics, veterinary costs, and comments from slaughterhouses were found when comparing PRRSV positive herd with PRRSV negative herds (Kristensen, C. S. et al., 2013b). Furthermore, some herds are discovered to have PRRSV by coincidence when a veterinarian takes annual routine blood samples. In addition, PRRSV does not always result in economical losses in a herd. However, for each PRRSV positive pig sold at 30 kg, the farmer has a loss of 20 DKK (Pig listing, 2016).

2.3 Incentives on national level to become PRRSV negative

A study has shown that PRRSV affects the Danish economy in a negative way with an estimated economical loss of 100 million DKK/year. This estimate is based on losses caused by acute outbreaks of PRRSV, chronic PRRSV infection, and slaughter and export related losses since slaughterhouses in Denmark have additional costs associated with PRRSV infected pigs (Kristensen, C. S. et al., 2013a). A number of international markets have banned the import of PRRSV infected meat even though spread of PRRSV through sale of pork meat can be considered non-existent (Pharo, H. et al., 2011). PRRSV positive herds must be reported to the authorities, and pigs from positive herds have to be slaughtered separately from pigs from PRRSV negative herds for up to 60 weeks from the time of infection. These separated slaughterings are meant to ensure and maintain Danish pork export, but it is associated with additional costs for the slaughterhouses (Kristensen, C. S. et al., 2013a).

2.4 PRRSV status in Danish swine herds

In Denmark, PRRSV status is monitored in approximately 3,000 SPF herds participating in the SPF-SuS who tasks is to manage and develop the SPF system and to declare the health status of all pig herds. SPF herds constitute about 40% of the total number of swine herds in Denmark. (Kristensen, C. S. et al., 2014c). An inventory of the most common SPF declared diseases shows that PRRSV is amongst the three major diseases in Denmark along with *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* (see Figure 5).

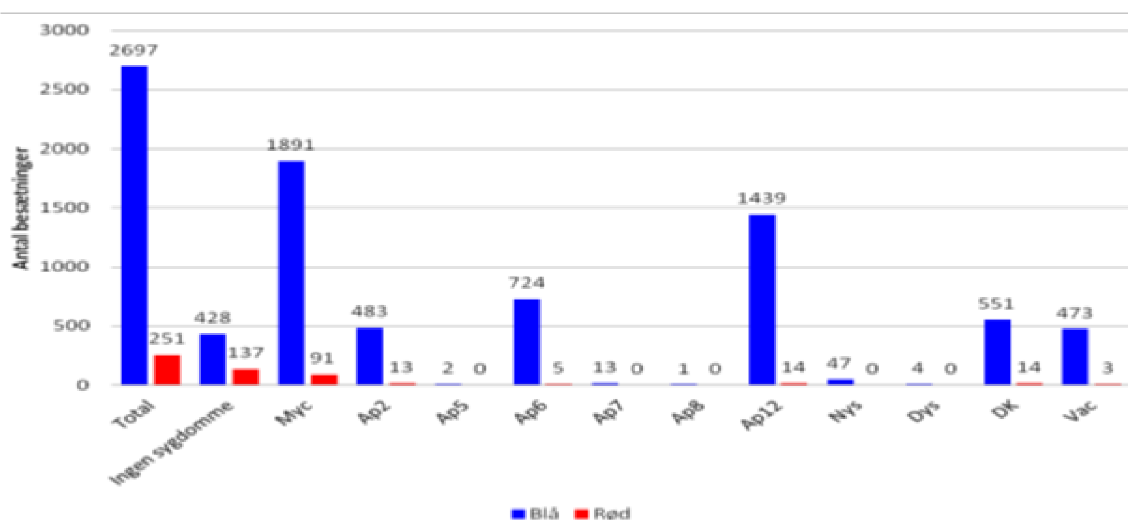


Figure 5. The distribution of SPF declared diseases in SPF herds. (Source: SPF-health management, 2015).

In red herds, which are a SPF breeding herd with the highest level of health status in Denmark (health control is performed once a month by VSP's department for health control), PRRSV type 1 ("DK") is found in 6% of the herds, whereas PRRSV type 2 ("Vac") is found in only 1%. This is somewhat different in the blue herds, which are a SPF production herd with same set of rules as the red herds but considerably less intense (health control is performed at least every 15 weeks of the specific herd veterinarian). In blue herds, both type 1 and type 2 are registered in about 20% of the herds. Furthermore, about 10% of the blue herds are registered with a mix of both type 1 and type 2 (SPF-health management, 2015).

Nonetheless, a positive development of PRRSV negative herds has been seen over the last few years. Since 2003, the proportion of PRRSV negative SPF herds in Denmark has increased and the same is likely to happen in herds that are not registered in the SPF-SuS. Since the number of herds sanitized for PRRSV (going from PRRSV positive to PRRSV negative) is lower than the number of herds being infected with PRRSV (going from PRRSV negative to PRRSV positive), the increase in PRRSV negative SPF herds are partly due to the cessation of PRRSV positive herds (Kristensen, C. S. et al., 2014b). In April 2015, 81% of the SPF herds were declared free of PRRSV type 1 and 84% of SPF herds were declared free of PRRSV type 2 (SPF-health management, 2015).

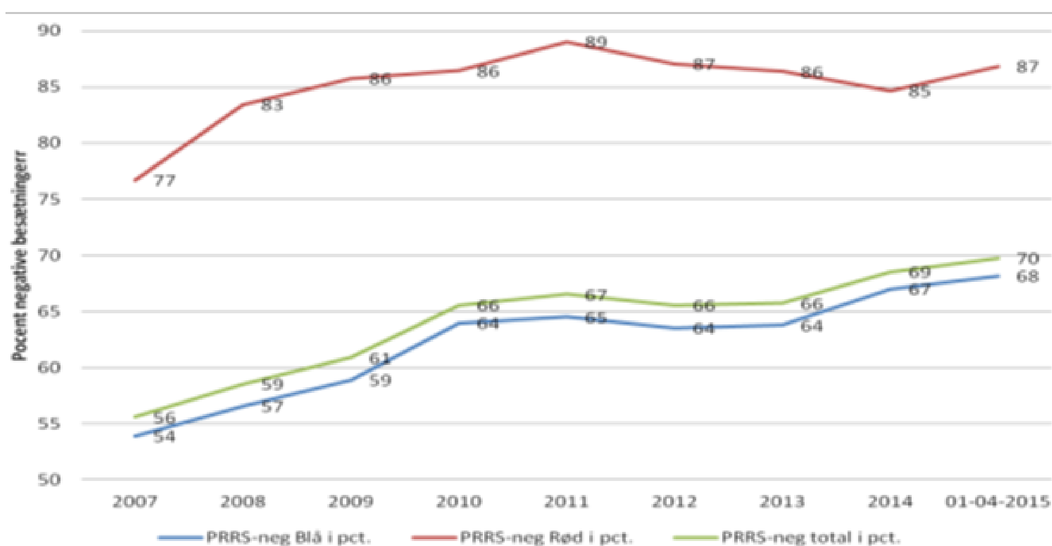


Figure 6. The development of the proportion of SPF herds declared free of PRRSV. Red line: SPF red herds. Blue line: SPF blue herds. Green line: All SPF herds. (Source: SPF-health management, 2015)

Figure 6 illustrates the development of the proportion of SPF herds declared free of PRRSV. SPF herds declared free of PRRSV (regardless of PRRSV type) has been steadily increasing due to PRRSV elimination from the herds. Of the most common declared SPF diseases, PRRSV is the disease most herds have tried to eliminate, showing a huge interest in being free of PRRSV (SPF-health management, 2015).

2.5 Vaccination

In an attempt to lower and control the impact and transference of PRRSV type 2, enormous efforts have been put into the production of vaccines (Shi, M. et al., 2010a). Several vaccines are available on the market. The vaccines can mainly be divided in two categories: (1) Consisting of products containing live virus derived from cell culture attenuates of a virulent field strain, e.g. the Ingelvac PRRS® MLV and Porsilis® MLV vaccines, (2) Consisting of products containing inactivated preparations of attenuated PRRSV strains, e.g. the Progressis® and PRRomiSe® vaccines (Murtaugh, M. P. et al., 2011). Due to high genetic diversity and drift, it can be difficult to create a vaccine with a satisfying protection level. Furthermore, a lack of cross-protection between strains in the existing vaccines is seen, and the vaccines have problems inducing the correct and optimal immune response (Nauwynck, H. J. et al., 2012). Experiments have shown that attenuated live vaccines have the ability to reduce the amount of viral shedding to the environment with less PRRSV load in oral fluids and reduced PRRSV in the air; thereby, the vaccine can be a method to enhance viral control (Linhares, D. C. L. et al., 2011). The use of attenuated vaccines in swine herds is a common tool, but caution needs to be taken since the attenuated virus in the vaccine poses a risk of reverting and becoming highly virulent, and extensive use can thereby contribute to increased diversity of PRRSV (Liu, D. et al., 2010). This scenario was seen in Denmark when Ingelvac PRRS® MLV vaccine was introduced, and a spread of vaccine virus to non-vaccinated sows happened. Furthermore, infection with the vaccine virus was also identified in several herds, where the vaccine had never been used (Bøtner, A. et al., 1997).

2.6 Control and elimination of PRRSV

When a herd is found positive for PRRSV, it is important to minimize the economic losses and maximize the production efficiency. In relation to this, different strategies can be implemented: (1)

Eliminate or remove all pigs from the herd (total sanitization), (2) eliminate or remove a specific age group e.g. weaners (partial sanitization), or (3) try to control PRRSV in such a way that sows are PRRSV antibody positive through vaccination and thereby wean PRRSV negative pigs. If the goal is total sanitization, nothing else has to be done in the herd, but if the goal is partial sanitization or try to control PRRSV transmission, it is essential that the herd is stabilized before the actual sanitization. A stabilized herd is defined as a herd where there is no clinical or diagnostic evidence of the virus, and the sows have antibodies against PRRSV. Therefore, no active circulation or transmission of PRRSV from sow to sow or pig to pig (horizontal transmission) or from sow to piglet (vertical transmission) occur. Furthermore, in a stabilized herd, weaners are PRRSV negative when tested (Mitchell Veterinary Services, 2014; Gillespie, T. G. et al., 2003; Rajic, A. et al., 2001). A herd can be stabilized using a modified live vaccine for mass vaccination (blitz vaccination) of the breeding animals (sows, gilts, and boars) (Gillespie, T. G. et al., 2003). Besides vaccination, several other factors are important to achieve a stabilized herd, for example the way gilts are recruited. Previous studies have shown that PRRSV exposed in a breeding herd can be controlled through good management of the gilt pool. Also, the level of exposure in a herd appears to decrease if the risk of introducing potentially viremic animals into the sow teams decreases (Dee, S. et al., 1995). The goal of gilt recruitment is to introduce PRRSV immunized gilts (negative for PRRSV) into the sow teams. Different strategies are used for gilt recruitment some of which are explained in the following.

The farmer can choose to buy gilts from PRRSV positive herds (same type of PRRSV), these gilts have to be minimum 5 months of age upon arrival. The gilts purchased from a PRRSV positive herd have to stay in quarantine for a minimum of 8 weeks, and before the gilts are introduced in the herd, blood samples are taken to make sure that the virus is not present in the gilts. Samples are tested with IPT and ELISA where a low or negative IPT is equivalent to low or no virus excretion, respectively, and a positive ELISA means that the gilts have antibodies against virus (immunized). After the quarantine period, the excretion of the virus is assumed to have ended or to be at a low level, and the gilts can be moved into the herd. However, this method is not very reliable since the gilts still are at risk of excreting PRRSV, and therefore, this method is not recommended (Nilubol, D. et al., 2002; Kristensen, C. S. et al., 2014a). Another approach is to buy gilts from a PRRSV negative herd. PRRSV negative replacement gilts quarantined prior to introduction into the

herd are of great importance for achieving control of PRRSV in infected herds.. The gilts are bought at an age of 3-4 months, so that the gilts have time to get infected and immunized "naturally" before moving into the sow team. For a minimum time period of 8-12 weeks, the gilts have to be in a quarantine stable with other pigs that excrete PRRSV. This strategy can be difficult, since not all gilts get infected in quarantine stable (Nilubol, D. et al., 2002). A third option for gilt recruitment is to recruit gilts from the original herd. Once again, it is important that these gilts are infected and immunized naturally in a quarantine stable before they are moved to the sow team. Based on previous experiences, regardless of whether the gilts originate from a PRRSV positive herd, a PRRSV negative herd, or the original herd, it is suggested to be a good idea to vaccinate the gilts with a MLV before introduction to the sow team to ensure similar immune status and a stabilized herd (Rajic, A. et al., 2001). The gilts should be vaccinated immediately after arrival to the quarantine stable and revaccinated 3 weeks later. They have to be in the quarantine stable for 12 weeks to make sure that no excretion of virus occurs. If possible, it is best to close the quarantine stable down for 12 weeks before new gilts are purchased (Kristensen, C. S. et al., 2014a).

2.7 Total sanitization

The safest method to eliminate PRRSV is to sanitize the whole herd by removing all pigs and thoroughly washing and disinfecting all sections. New PRRSV negative pigs can be introduced into the herd after three weeks (Andreasen, M., 2000).

2.8 Partial sanitization

Partial sanitization can be done using a MLV. It has been shown that through this strategy, the sows become immune and wean PRRSV negative piglets within 4-6 months (Rajic, A. et al., 2001). Usually, spread of virus originates from parts of a herd where there is no sectioning, which is most likely in the finisher unit. Partial sanitization consists of 2 phases, and only the sections where there is a continuous infection of young pigs need to be emptied (see Figure 7) (Gillespie, T. G. et al., 2003).

Phase 1 (the stabilization of the sow team) takes place before the actual sanitization and aims to get PRRSV under control in the sow team (stabilized sows). An important measure to take is to recruit the gilts as described earlier, and optimize management routines. In order to get a stabilized sow team, a "homogenization" of the pigs is needed. Homogenization refers to the creation of

a uniform pig population, where every pig has immunity against the PRRSV (a stabilized herd). A strategy to achieve homogenization is to use a MLV. All of the sows, gilts and boars are vaccinated twice with one month apart (blitz vaccination), and sometimes the offsprings are vaccinated as well. The production of PRRSV negative piglets will occur at earliest 5 weeks after the latest vaccination (Mitchell Veterinary Services, 2014; Gillespie, T. G. et al., 2003). The purpose is to get a stabilized sow team that weans PRRSV negative piglets. Vaccinated herds are more likely to remain stable than non-vaccinated herds, and thereby, the vaccinated herds can produce PRRSV negative pigs (Rajic, A. et al., 2001). Another phase 1 strategy is called "load, close and homogenize". This is an even safer method than just vaccinating (homogenization), because no new animals enter the herd, and thus, the risk of introducing PRRSV into the herd is avoided. The steps in this strategy (load, close and homogenized) are described in the following (homogenization is already described):

- Step 1 (load) consists of packing the herd with gilts obtaining enough pigs to maintain the production of piglets during the time the herd is closed. All of the newly purchased gilts are PRRSV negative and will be a part of the stabilization strategy in the closed herd.
- Step 2 (close) consists of closing the herd to all new entries. The herd is closed as long as it takes to achieve immunity against PRRSV on the herd level and thereby get a uniform population. Furthermore, it has to stay closed until the transmission of the circulating PRRSV is stopped in the herd. Generally, a recommendation will be to close the herd for a minimum of 200 days, but it can vary depending on various factors such as management, protocols, etc., which dispose a risk to the circulation of PRRSV. Overall, this step is to minimize the introduction of a new PRRSV type into the herd, while the pigs are developing immunity (Torremorell, M. et al., 2000; Mitchell Veterinary Services, 2014).

The actual sanitization takes place in phase 2. The herd sanitization has to take place in the sections where an active spread of virus is happening. These sections are emptied, cleaned and disinfected. The removal of pigs may involve finishers and/or nursery pigs. A way to empty the nursery is by selling the weekly batch of pigs, which should have been inserted in the unit. (Gillespie, T. G. et al., 2003). Different methods can be used:

- Farrowing stop is recommended especially in those herds where it can be difficult to determine if the sows wean PRRSV negative piglets. This usually takes place in herds selling pigs at 7 kg or if there is a continuous flow in the nursery (not AI/AO). However, farrowing stop cannot compensate for the lack of PRRSV stability in the sow team (Andreasen, M. et al., 1997). A farrowing stop means one week with no pigs in the unit. By law (Bekendtgørelse om beskyttelse af svin nr. 17 af 07/01/2016 §35), the piglets have to stay with the sow for at least 4 weeks. In this way, the farrowing unit needs to be empty for 5 weeks in total (Kristensen, C. S. et al., 2014a).
- If the ongoing infection is circulating in the nursery, the herd can decide to depopulate this unit. If the nursery has an ongoing infection of PRRSV, there is a risk of the sows being infected/reinfected. A recommendation is to depopulate the nursery unit if it is located close to the sow unit and furthermore to depopulate it 3 months before a farrowing stop (Andreasen, M. et al., 1997). A way to depopulate the nursery section is to move the weaners to a different location or by selling pigs at 7 kg. Studies have shown that depopulation of the nursery can lead to PRRSV negative pigs and improvement in the mortality and average daily weight gain (Dee, S. A. et al., 1994). If the depopulated weaners are moved to another location, this location cannot be placed near the herd. S. A. Dee et al. 1994 showed that 5 out of 7 herds still had PRRSV positive pigs after depopulation if the new location where less than 30 meters away from the cleaned nursery. Furthermore, the study suggested that it makes no difference whether the nursery unit is empty for 7 or 14 days after cleaning and disinfection. This information is advantageous in those situations where it is impossible due to logistic reasons (for example lack of space) for the farmer to have an empty nursery for a longer time period (Dee., S. A. et al., 1997). If the nursery is fully sectioned, it can stop the spread of PRRSV between the weaners, and in some herds, this can be enough in order to become PRRSV free without having to depopulate (Andreasen, M. et al., 1997).
- If the ongoing infection is in the finisher unit, this unit can be depopulated. A way of doing this is to move the finishers to a different location, sell the pigs at 30 kg, or slaughter the pigs. Experiments have shown that partial depopulation within the fin-

isher unit, where only some parts of the section are emptied, cleaned and disinfected, is not recommended because this strategy has a much higher risk of reinfection. Therefore, the safest method is to totally depopulate the finisher unit (Hassing, A. G. et al., 2000).

When the respective sections with infection are emptied, cleaned and disinfected, PRRSV negative pigs can be introduced into herd. These pigs can be monitored with blood samples to see if they have responded or not (Gillespie, T. G. et al., 2003).

A herd can decide not to eliminate PRRSV but instead have a PRRSV positive herd with a stabilized sow team (sows with antibodies against PRRSV). In this way, the herd weans PRRSV negative pigs. If the herd is located near other swine herds, and especially if these herds are PRRSV positive, the risk of becoming reinfected with airborne PRRSV is high. To examine this risk, a GIS map can be helpful. This is a topographical map with the herd investigated placed in the center and other herds and their SPF status within 3 km of the investigated herd placed in the periphery (Mortensen, S., 2001).

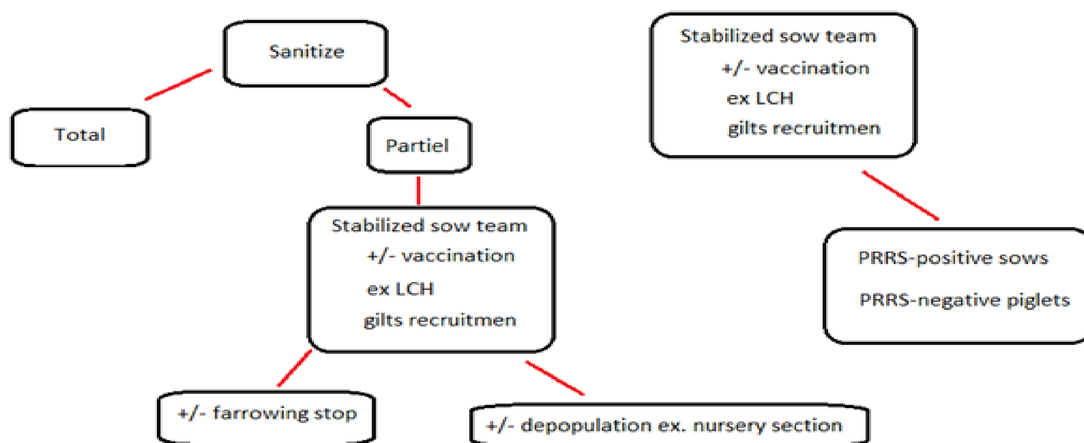


Figure 7. Schematic view of different approaches to sanitize a herd. source: Original work by the authors.

2.9 Appropriate herd building and pig flow

Previous experiments have shown that PRRSV can be transferred between units, and that airborne transmission between units located near each other often occurs (Kristensen, C. S. et al., 2002). To prevent contamination between different age groups, the sections must be separated completely from each other with full sectioning. Full sectioning can reduce air transmission much more than

partial sectioning, because partial sectioning shares airspace between the individual sections, which results in greater transport of particles between the sections. The herd must be constructed in a way where the sow team is at one end, and nursery and/or finisher unit are in the other. Furthermore, the pigs must be transported through the herd without crossing a section they have been in previously. An example of bad herd building and pig flow is if the nursery section has direct access to the farrowing unit. All pigs leaving the nursery section have to be transported through the farrowing unit on their way to the finisher unit. In this way, weaners can reinfect sows constantly, and it is thereby difficult to achieve PRRSV stability in the sow unit (Andreasen, M. et al., 1997).

2.10 Management

Whether a herd simply wants to remain stable with a stabilized sow team, or whether they want to be partially sanitized, the herd has to implement some management strategies. Focus on management can contribute to the success of control/elimination of PRRSV. One management tool that can be implemented is the McRebel (Management changes to Reduce Exposure to Bacteria and Eliminate Losses) strategy. This tool can be implemented at the same time as the herd tries to stabilize the sow team. The purpose of McRebel is to make a systematic approach to reduce the spread of secondary bacteria and PRRSV in the farrowing and nursery units. In this way, the herd can optimize the health and growth of the pigs and reduce the mortality in both the farrowing and nursery units. The concept of McRebel is that an optimization of suckling piglets' growth requires minimized intervention and maximized supportive care. The main focus in McRebel is to have as many piglets remaining with their birth mother, or to have as many piglets remaining with the mother from which they got the colostrum. Furthermore, the principles of AI/AO (all in/all out) have to be followed in the litters. The protocol of McRebel is as follows:

- Cross-fostering may only occur during the first 24 hours of the piglets' life. It is important only to move the minimum of piglets necessary to load functional teats. The smallest piglets are given the lowest priority; larger and more vigorous piglets are prioritized when assigning functional teats. Finally, cross-fostering with the purpose of creating uniform or single gender litters is not allowed.
- It is not allowed to move the piglets between different sections (farrowing and nursery units/sections). The herd must follow strict AI/AO production. Experiments have

indicated that it is possible to prevent an outbreak of airborne virus if the pigs are inserted into sectioned units with AI/AO. Other results have showed that pigs are at a higher risk of being infected in the nursery unit if sectioning is inadequate (Jensen, T. et al., 2001).

- Eliminate the use of nurse sows.
- If a piglet cannot be weaned due to sickness, poor body condition, etc., this piglet has to be removed from the production system. The piglet should not be transferred back to sections containing younger litters. If the piglet does not recover, the piglet should be eliminated. However, sometimes it is better to euthanize unhealthy piglets then try to cure them.
- Minimize handling of piglets with e.g. antibiotics and iron injections. Infection can be transferred to susceptible pigs through poor needle hygiejne. Thus, it is important to change needle between pens.
- Create a good environment in the nursery unit to maximize survival. It is recommended that the smallest piglets are placed in a warm, non-drafty part of the unit.

Implementing McRebel strategy would be at a minimal cost, and the strategy can be used while attempting to stabilize a herd. Studies have shown that by implementing McRebel principles, a significant reduction in piglet and weaner mortality as well as a significant increase in mean sale weight were seen compared to before implementation (McCaw, M., 2003; Dee, S. et al., 1996; Otake, S. et al., 2001; McCaw, M. B., 1999).

3 Laboratory techniques

In the following sections, the theoretical background for the used laboratory techniques and approaches will be described in detail.

3.1 Conventional PCR

PCR is a detection method, which is able to exponentially amplify DNA of a specific target gene fragment in vitro to such an amount that the DNA can be investigated. It is used to detect the presence of a certain DNA fragment (target), and the procedure is carried using a specific master mix consisting of specific primers (forward and reverse), a DNA-polymerase, deoxyribonucleotide tri-

phosphates (dATP, dCTP, dGTP, and dCTP), DNA template, and a mix of buffers and salts (the water solution). After the master mix has been made, the process consists of three main steps (see Figure 8):

1. Denaturation of the DNA strand, producing 2 single-stranded DNA fragments. This procedure occurs at a temperature of 95 ℃.
2. Annealing where the primers hybridize to each end of the targeted single-stranded DNA fragment. The temperature of this process depends on the primers' melting point but is carried out around 45-60 ℃.
3. Elongation of the primers by the DNA-polymerase attaching the nucleotides adenine (A), thymine (T), cytosine (C), and guanine (G) to their corresponding base pair. This process takes place at a temperature around 70 ℃.

These three steps will be repeated about 40-45 times, and the amount of targeted DNA will then have increased exponentially due to the fact that every new fragment can be used as a template. To multiply the DNA segments, a DNA-polymerase is needed. The DNA-polymerase originates from *Thermus aquaticus* (Taq). This specific polymerase makes it possible to accomplish PCR in elevated temperatures, which makes it less likely for mismatched annealing to non-target sequences to occur (Arnheim, N. and Erlich, H., 1992).

The primers play a vital role as they are responsible for targeting the right DNA sequence. Primers are designed for every target. Ideally, primers should have a CG nucleotide content over 50%, have a length of at least 20 nucleotides, and have an annealing temperature of around 50-60 ℃. Furthermore, the primer region should be found in the most conserved region of the target genome, so that the primer cannot mismatch. However, PRRSV which has a very changeable genome, it can be necessary to add several primers to be sure to find the virus searched for (Arnheim, N. and Erlich, H., 1992; Strachan, T. and Read, A., 2010a).

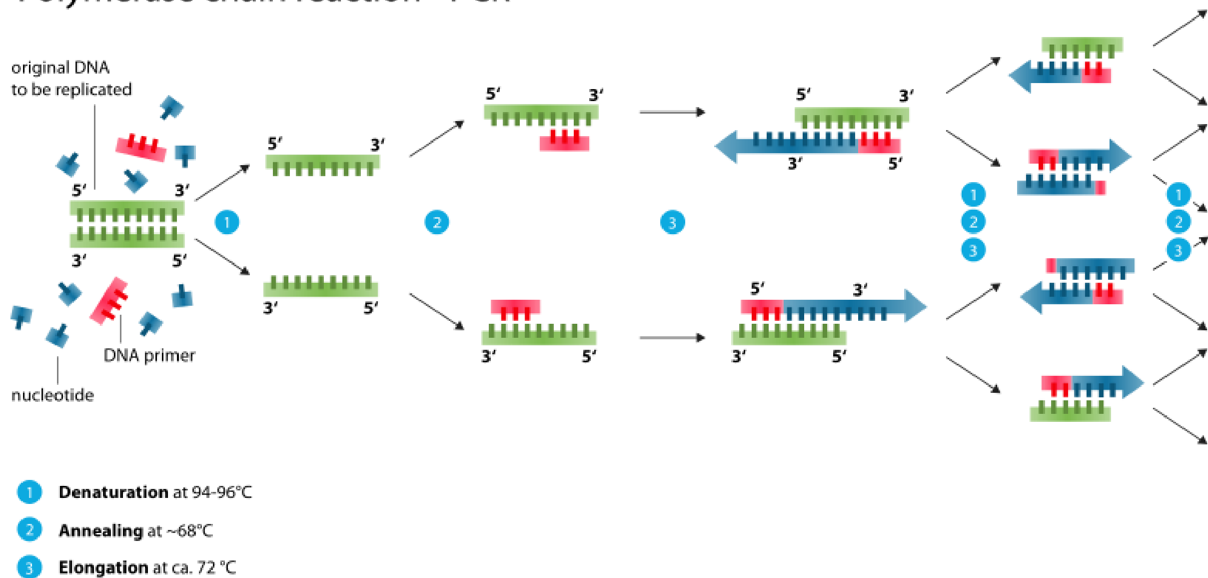


Figure 8. PCR reaction steps. (Source: PCR reaction, 2016).

3.2 Real time RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) is used for RNA viruses, where the single-stranded RNA is firstly reverse transcribed into complementary DNA (cDNA). Specific primers and probes designed to find specific targeted areas on the cDNA are then added. Undergoing the same PCR steps, new copies of the targeted area are made by complementary base pairing throughout 45 thermal cycles. This process ultimately creates enough copies of the target region to become detectable and either gives a positive or a negative result (qualitative data/result). The real time RT-PCR version furthermore provides quantitative information in "real time" meaning that after every amplification cycle, it is possible to detect the amount of products produced. The amount of products are detected through labelling with a fluorophore. The fluorophores emit a light signal for each sample, and this signal is measured throughout every cycle of the real time RT-PCR reaction with the amount of light correlating with the amount of total DNA present in the sample after each amplification cycle (see Figure 9). The fluorescent signal has a set threshold, and the cycle at which it crosses this threshold is called cycle threshold (Ct). This threshold can be reached quickly if there is a large amount of target DNA present at the start of the reaction. A large amount of target DNA present at the start of the reaction would require fewer cycles to reach the number of target products

sufficient to generate a fluorescence signal - that is, the Ct-value is lower. Furthermore, if the Ct-value is over 40, which is a high Ct-value, the sample screened would be considered negative for the searched target. A great advantage of the real time RT-PCR is also that it is in a closed system as opposed to conventional PCR; thereby, there is less chance of contamination. Possible background noise is envisioned using a non-template control (NTC) (Kubista, M. et al., 2006; Strachan, T. and Read, A., 2010; Basic Principles of RT-qPCR, 2004).

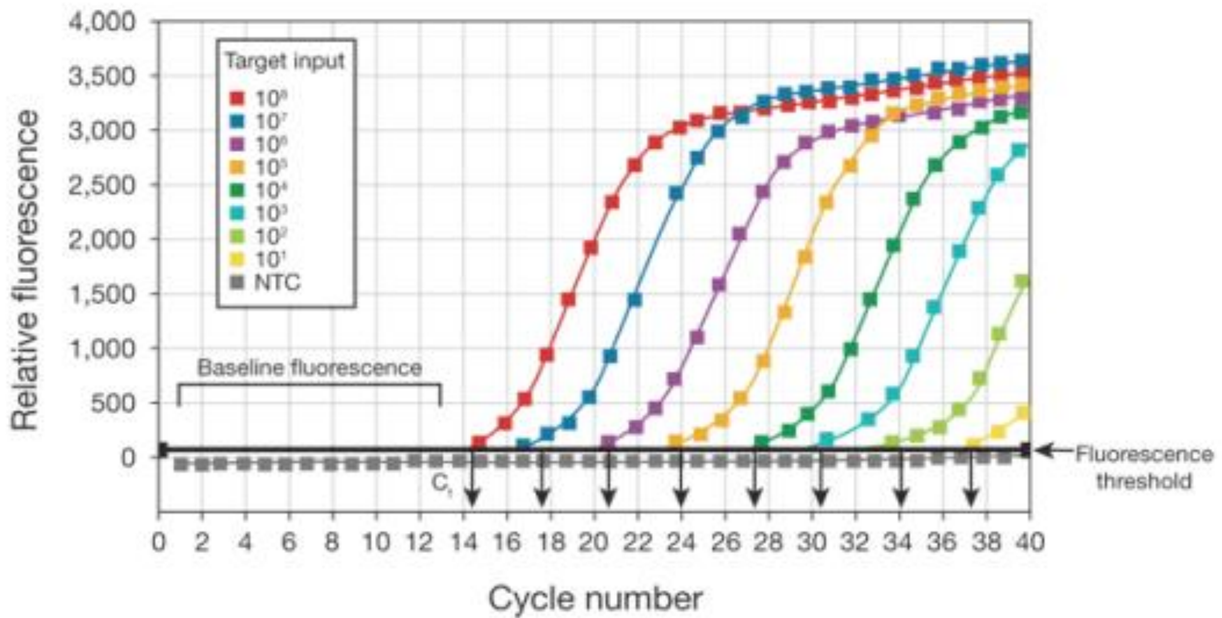


Figure 9. Relative fluorescence vs. cycle number. Amplification plots are created when the fluorescent signal from each sample is plotted against cycle number. Amplification plots represent the accumulation of products during the cycles of real-time PCR. The samples used to create the plots in this figure are dilution series of the target DNA sequence. (Source: Real time PCR, 2016).

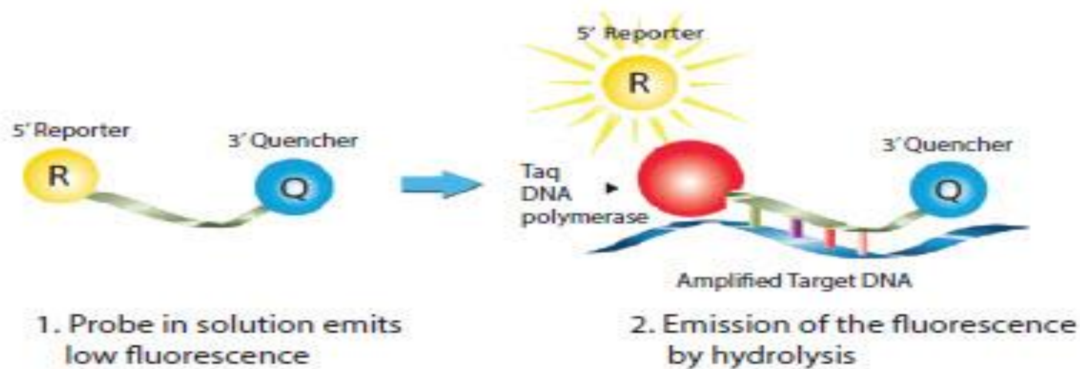


Figure 10. Dual labeled probe chemistry. (Source: Dual labeled probe, 2016).

3.3 Real time RT-PCR: Dual labeled probe chemistry

The fluorescence activity in real time RT-PCR described earlier is based on so-called dual labeled probe chemistry (see Figure 10). The chemistry underlying this PCR method is based on the thermostable Taq DNA-polymerase, which edits 5' to 3' exonuclease activity. This technology uses a dual labeled non-extendable probe and two primers specially designed to find the targeted section in the target genome. The probe contains a fluorescence reporter dye at its 5'-end and a quencher at its 3'-end. When the probe is attached to the target region during PCR, the quencher suppresses the fluorescence of the reporter by absorbing the light from the reporter. During the amplification phase, the probe is exchanged with dNTPs. During the elongation step with the 5' to 3' exonuclease activity, Taq DNA-polymerase hydrolyzes the probe. Thereby, the quencher and the fluorescence reporter dye separate, and the quencher is left unable to suppress the fluorescence reporter dye. This leads to a continuously increasing amount of light being emitted during PCR cycles (Holland, P. M. et al., 1991; Gibson, U. E. M. et al., 1996). The technique can be used as a multiplex assay, where specific primers and probes are added to fit the targeted sequence (Bustin, S. A. et al., 2000).

3.4 Target sequence

To determine the presence/absence of a specific pathogen, it is important for the assay that it is targeting a highly conserved area of the genome of interest. Localizing the right target sequence can be difficult with RNA viruses (such as PRRSV) since the mutation rate is very high (Chang, et al., 2002). For PRRSV, the target sequences are ORF6 and ORF7, which are considered to be rather conserved areas in PRRSV genome (Balka, G. et al., 2009). Because PRRSV consists of two genotypes (type 1 and type 2), a multiplex quantitative real time RT-PCR assay including primers and probes for both types is used (Bustin, S. A., 2000). A PRRSV type 1 and 2 multiplex real time RT-PCR assay has been validated before (Kleiboeker, S. B. et al., 2005; Wernike, K. et al., 2012).

3.5 DNA sequencing

DNA sequencing is the gold standard when it comes to screening a genome for new mutations (Strachan, T. and Read, A., 2010b). Several new and very fast sequencing methods have been developed since the dideoxy (Sanger) sequencing method ("first generation sequencing"). These are called "Next-Generation Sequencing" (NGS). The NGS methods can make massive parallel anal-

yses with a very high output at a very low cost (Strachan, T. and Read, A., 2010c). Examples of NGS methods are:

- Illumina (Solexa), sequencing by synthesis of PCR amplified DNA
- Roche 454, which uses pyrosequencing
- Ion Torrent, a semiconductor proton/personal genome machine (PGM) sequencing
- SOLiD, sequencing by ligation of PCR amplified DNA
- Single molecule real time sequencing

In this thesis, these two sequencing methods will be described in detail: Sanger sequencing and the NGS method Ion Torrent (semiconductor proton/PGM sequencing).

Sanger (dideoxy) sequencing (see Figure 11) is also called the chain termination method and relies on dideoxynucleotides (ddNTPs), a type of deoxynucleoside triphosphates (dNTPs) where the hydroxyl group at the 3'-end is replaced by a hydrogen atom. During sequencing, when the ddNTPs bind to the DNA strand, the sequencing process terminates and no other dNTPs can bind. In order to perform the sequencing, primers are added to a solution containing the targeted DNA sequence, and the solution is then divided into 4 different PCRs which each contain one of the four ddNTPs A, T, C or G. When the four PCRs are terminated, DNA segments of various lengths are obtained because replication terminates randomly. Then solutions from the four PCRs are run through a denaturing polyacrylamide-urea gel, each PCR solution in its own lane. This results in many different bands on the gel illustrating the different sized fragments, which can then be pieced together creating the right order of the nucleotides in the investigated target genome (Sanger sequencing, 2016).

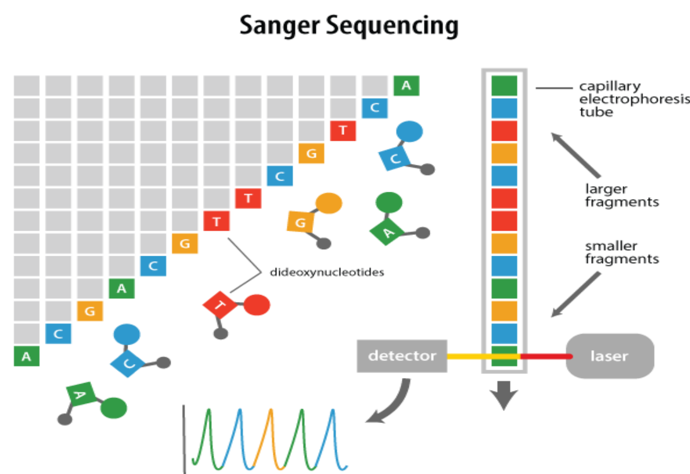


Figure 11. Illustration of Sanger sequencing. (Source: Sanger sequencing illustration, 2016).

Ion Torrent semiconductor sequencing (see Figure 12) is a NGS method using emulsion PCR based on a single library template bound to a microbead which is captured in an oil droplet containing DNA-polymerase, primers and dNTPs. The method exploits that a hydrogen ion is released when a dNTP is incorporated in the DNA strand by the DNA-polymerase. The droplet fit into a microwell or a glass plate inside a microchip, where the amplification of the template takes place. The sequencing is measured on an Ion Torrent Personal Genome Machine sequencer which relies on a high density array of micro-machine wells performing nucleotide combination. Every well contains different DNA templates, and beneath the wells is an ion sensitive layer and underneath that, a proprietary ion sensor. The microchip is flooded several times with one nucleotide at a time, and if the DNA-polymerase incorporates a nucleotide, a hydrogen ion is released. When a hydrogen ion is released, the pH of the solution decreases and this change is detected by the ion sensor. In addition, the hydrogen ions are detected on an ion semiconductor sequencing chip. Furthermore, if there are two identical bases, the signal is doubled. This technique directly connects a chemical reaction with a digital output. The Ion Torrent PGM generates a data output of approximately 10 to 1,000 megabytes depending on the used type of ion semiconductor sequencing chip (Ion Torrent semiconductor sequencing, 2016). Prior to Ion Torrent sequencing, a library preparation of the specific DNA or RNA of interest is necessary. Hereby, the sequences are split into fragments, and the adaptors are ligated to the ends of the fragments (Metzker, M. L., 2010; Bexfield, N. et al., 2011; Bahassi, E. M. et al., 2014).

The raw data obtained from the NGS sequencing has to undergo a process where all adapter sequences and low quality reads are erased. After this procedure, the reads can be compared either to a reference sequence, or they can be assembled de novo. The consensus sequence produced from the assembly can then be compared to a database to compare the sequence to already existing sequences. Such a database could be the GenBank of NCBI (the Basic Local Alignment Search Tool (BLAST)). NGS can be used on amplicons with already available sequences, but it can also be of help in de novo sequencing. This is very useful for investigating newly formed virus (Metzker, M. L., 2010; Bexfield, N. et al., 2011; Bahassi, E. M. et al., 2014).

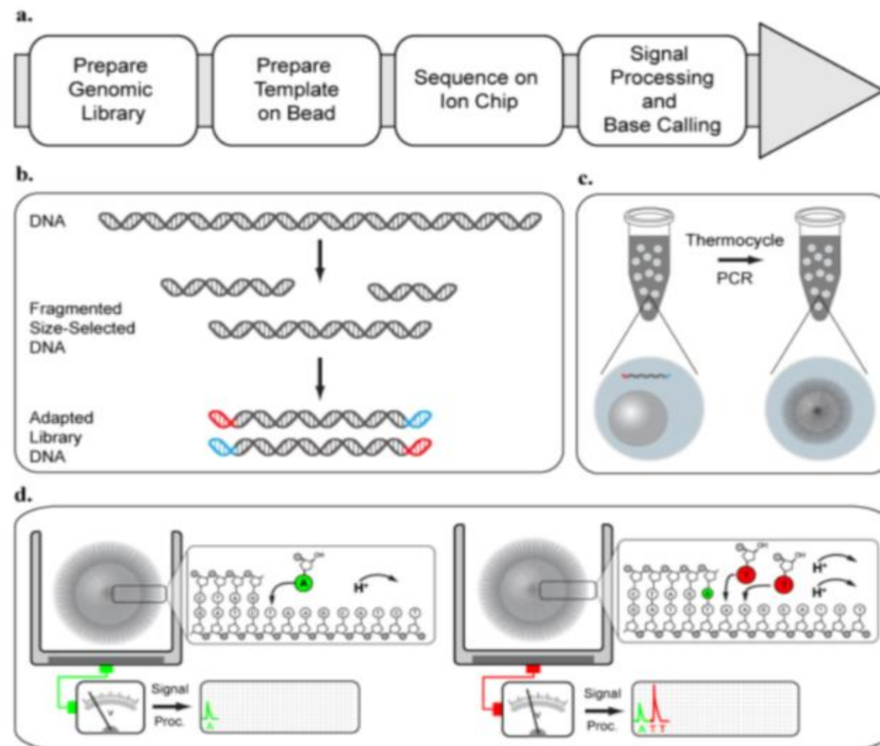


Figure 12. The principle of Ion Torrent semiconductor sequencing. (Source: Ion torrent semiconductor sequencing, illustration, 2016).

Methods and materials

This master's thesis consists of two focus areas: (1) A management related observational study, where we investigated clinical symptoms and management strategies in the herds, and (2) a virus detection and genetic characterization study focusing on sample collection and genetic analysis.

1 Herd selection

Three Danish swine herds were selected in close cooperation with their responsible veterinarian. The herds had to fulfil the following criteria:

1. The sow teams had to be stable with no circulation of PRRSV within the farrowing unit.
2. The herds had to be sought sanitized with blitz vaccination of the sows using Boehringer Ingelheim's Ingelvac PRRS® MLV and implementation of specific management strategies.

3. The herds had to have an ongoing problem of PRRSV in weaners.
4. To reduce the amount of bias, the herds had to be of similar type, e.g. similar size and production system.
5. Organic ("økologisk") herds were excluded.

In order to ensure that criterion 3 was fulfilled, 20 blood samples were collected in each herd to test for the presence of PRRSV. Idexx ELISA was used for virus detection. The samples were collected from 30 kg pigs (10-12 weeks of age) to avoid the presence of maternal antibodies. PRRSV positive subjects were found in each herd suggesting that virus is circulating. All of the three herds were located in Jutland, Denmark.

1.1 Herd description

In the following, descriptions of the three chosen herds are given. This information is based on personal communication (e-mails) with the farmers as well as answers from the questionnaire (see Appendix 1).

1.1.1 Herd 1

Herd 1 is a blue SPF herd with *Mycoplasma* (Myc) and PRRSV type 2. The herd is a production herd with 1,500 sows and sells 30 kg pigs. The problems with PRRSV started in 2012. However, no clinical signs of PRRSV were observed. PRRSV elimination was attempted in January 2013. All sows were vaccinated with 2 ml Ingelvac PRRS® MLV, and all pigs in the nursery were vaccinated with 1 ml Ingelvac PRRS® MLV. The nursery was depopulated, washed and disinfected, and it was empty for 4 weeks. Sows were vaccinated twice a year with the latest vaccination (blitz) in May 2016 (piglets/weaners not vaccinated that time). The herd is sectioned and has a quarantine stable for the gilts off-site.

The herd produces their own gilts. When the gilts weigh 35 kg, they are sent to an off-site quarantine stable. When the quarantine stable is full, gilts are vaccinated and revaccinated 2 weeks after (2 ml ml Ingelvac PRRS® MLV). The gilts stay in the quarantine stable for 14 weeks after the first vaccination.

1.1.2 Herd 2

Herd 2 is a blue SPF herd with PRRSV type 2. The herd has 700 sows and mainly produces 30 kg pigs. However, due to the lack of space, the herd sells pigs at 7 kg (3,500 pigs) and 10-16 kg (5,800 pigs). The owner bought the herd in 2012 and vaccinated all newly acquired animals (gilts and sows). The problems with PRRSV started in 2014, mainly in the weaners. The observed clinical signs were mainly weak, longhaired and inactive weaners with swollen eyes. Many of the weaners were euthanized due to sequelae like sepsis, severe arthritis, and unthrifty health. The piglet mortality increased to 22%. Furthermore, weaner mortality increased to 8%. The herd started to eliminate PRRSV in spring 2016. All sows were vaccinated and revaccinated 4 weeks (2 ml Ingelvac PRRS® MLV). In addition, piglets (at day 10-12) were vaccinated in April 2016 (1 ml Ingelvac PRRS® MLV), but piglet vaccination stopped when the farrowing unit was sectioned in June 2016. All pigs that were not vaccinated in the farrowing unit were vaccinated in the nursery unit. At the same time that vaccination was carried out, implementation of different management strategies (McRebel) took place (see Appendix 2).

All gilts were purchased from a PRRSV negative herd at an age of 12-23 weeks. The farmer purchases 95 gilts every 13th week and vaccinates them upon arrival to the quarantine stable (on site) and revaccinates them after 3 weeks (2 ml Ingelvac PRRS® MLV). Gilts are placed in the quarantine stable for 8 weeks, before they are introduced into the herd. There are still clinical signs of PRRSV in the herd (unthrifty pigs and respiratory distress), but they are not that pronounced.

1.1.3 Herd 3

Herd 3 is a conventional herd, which implies that it is not SPF and therefore not declared free of some specific pathogens (SPF-Denmark, 2016). It is a production herd with 1,100 sows selling pigs at 30 kg. PRRSV problems started in 2002-2003. The observed clinical signs were mainly abortions in the sow unit. The herd started to eliminate PRRSV years ago. All weaners were vaccinated throughout 4-5 months (1 ml Ingelvac PRRS® MLV). In April 2015, the sows were vaccinated and revaccinated 4 weeks later (2 ml Ingelvac PRRS® MLV).

All gilts are purchased from PRRSV positive herds (PRRSV type 1 and type 2) at an age of 21-24 weeks. Gilts are vaccinated against both PRRSV type 1 and type 2, when they are 10-12 weeks old. Upon arrival, gilts are not quarantined. Clinical signs of PRRSV are still present in the

herd (unthrifty pigs, respiratory distress, and other sequelae among the weaners), yet no abortions in relation to PRRSV are seen.

2 Management related observational study

2.1 Registration of clinical symptoms

As described earlier, the clinical picture of PRRSV and other secondary infections can be longhaired and anorexic pigs (unthrifty), pigs with respiratory distress such as sneezing, coughing, dys- and hyperpnea, and mucus from nose and eyes, and worsening of diarrhea (Vestergaard, K. et al., 2007; Rossow, K. D. et al., 1994). The clinical signs of PRRSV vary a lot from herd to herd regardless of virus type (Kristensen, C. S. et al., 2014a). To investigate if observed clinical symptoms can give an idea of PRRSV status in a herd, a clinical registration scheme was developed. The registrations were completed in all three herds at age groups 4, 8 and 12 weeks (the same age groups where blood samples were collected). This scheme had three focus areas: (1) Unthrifty pigs, (2) pigs showing respiratory distress, and (3) blobs of diarrhea in the pens. Note that the observations were not done at the same time as the blood samples were collected but a week later, when the tonsil swabs (week 0) were collected.

2.2 Questionnaire

To evaluate management routines in the herd, a questionnaire was developed and e-mailed to the three herds. The questionnaire included questions regarding mortality, use of antibiotics, hygiene (change of needle, instruments for castration), washing and disinfection of the sections, cross-fostering strategy, and all in/all out principles. The questionnaire is based on the farmer's subjective answers and perceptions. The questions are mostly closed, however, a few of them are semi-opened or opened. The questions/answers are qualitative variables and cannot be measured.

The answers are used for assessing the herds' management strategies, which could give a better understanding of why PRRSV is persistent in the herds.

3 Viral detection and genetic characterization

3.1 Sample processing and pooling

For PRRSV detection in the herds, we collected blood serum, PUCS, tonsil swabs, and air samples at different age groups. Due to economical reasons, blood serum samples were pooled (5 or 10

samples according to age group) for further analysis. In addition, tonsil swabs were also pooled (5 samples). PUCS samples and air samples were tested individually (single/individual samples).

3.2 Sample size

The prevalence of PRRSV is assumed to be approximately 0-10% in each litter with increasing prevalence as age increases (Duinhof, T. F. et al., 2011). The three herds have an average of approximately 50 farrowings per week. The sample size is based on the assumption that each sow gives birth to 12 living piglets and is calculated in Microsoft Office Excel (see Appendix 3). The assumed prevalence p is 10%, the population size N is 600 (50 farrowings per week with 12 living piglets per farrowing), and the probability of finding at least one PPRSV positive pig P is 95%. Thereby, we get a sample size of 28. However, the prevalence varies between 0-10%, and for that reason, we chose a sample size of 60 in the younger pigs (0 and 4 weeks of age) and 30 in the older pigs (8 and 12 weeks of age). The smaller sample size in the older age group is due to the assumption that the prevalence of PRRSV is higher among the older pigs.

3.3 Sampling strategy

Selection of sample pigs was done randomly to provide a "true" picture of the PRRSV problem in the herds.

PUCS samples were collected from pigs at 0 weeks of age; we assumed that the prevalence of the PRRSV in the PUCS is very small so we collected all the placentas we could get. The staff collected all the placentas from one farrowing team and placed them in plastic bags in a refrigerator.

Tonsil swabs were collected from pigs at 0 and 12 weeks of age. Piglets at the age of 0 weeks were found in 12 pens, where we selected the youngest piglets in the herd (all younger than 48 hours). In each of these 12 pens, five tonsil swabs were collected from every second or third piglet (depending on the total number of piglets in that pen). Pigs at the age of 12 weeks were chosen from every second pen until 6 pens were found. In each af these 6 pens, five pigs were collected from every second or third pig (depending on the total number of pigs in that pen).

Blood serum samples were collected from pigs at 4, 8 and 12 weeks of age. Piglets at the age of 4 weeks were chosen from every second pen until 6 pens were found. In each of these 6 pens, five pigs were collected from every second or third piglet (depending on the total number of piglets

in that pen). Pigs at the age of 8 weeks were chosen from every second pen until 6 pens were found. In each of these 6 pens, five pigs were collected from every second or third pig (depending on the total number of pigs in that pen). However, in herd 3, which only had 5 pens available, 6 pigs were collected from every second or third pig in each of those 5 pens. Finally, the exact same pigs at 12 weeks of age that were selected for tonsil swabs were also selected for blood serum samples. Therefore, we could compare tonsil swabs and blood serum samples from the 12 week pigs.

3.4 Sample material, collection and handling

All samples were obtained from the 3 herds during several visits in October 2016 (see Table 1). The staff was instructed in how to collect the placentas (directly after farrowing, stored in clean plastic bags in a refrigerator). Between handing of each placenta, new gloves were used to avoid cross contamination. The PUCS samples were collected from all placentas within a maximum of 48 hours. The placentas were removed from the bag, turned inside out, and the umbilical cord remnant identified. A minimum of 5 cords were found from each placenta, and blood was milked into a tube (minimum 3 ml) (see Figure 13).

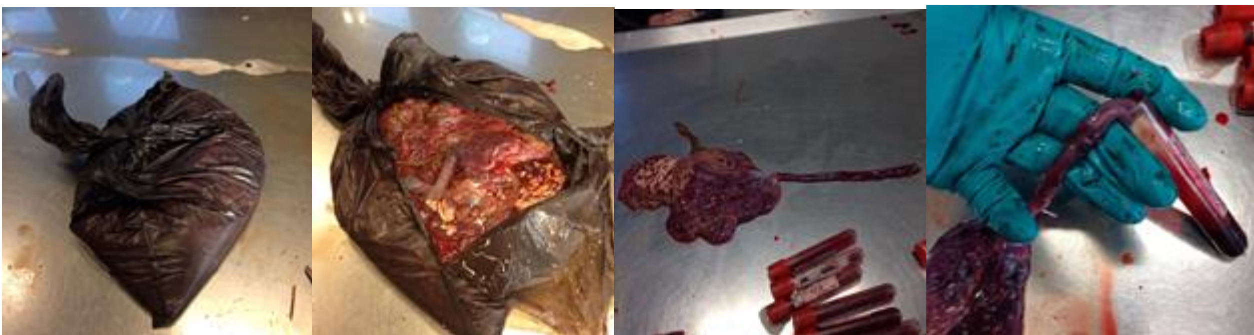


Figure 13. PUCS handling. (Source: Original pictures from the authors).

A total of 120 blood serum samples was collected in each herd (60 samples from 4 weeks old pigs, 30 samples from 8 weeks old pigs, 30 samples from 12 weeks old pigs). Blood was collected from v. jugularis externa through restraint by either fixating the smallest pigs on their backs or using a snare for the older pigs. The blood samples contained approximately 3-5 ml and were kept in a vacutainer for serum.

Tonsil swabs were obtained from 90 pigs in each herd (60 samples from 0 week old pigs, 30 samples from 12 weeks old pigs). The tonsil swabs were obtained by restraining the pig either manually (if <10 kg) or with a snare (if >10 kg) and then opening the mouth with a gag. The tongue was then pressed down, and the swab sample was taken by touching the rough area in the oropharyngeal area for about 3 seconds. The swab was placed in a plastic container with phosphate-buffered saline (PBS) and refrigerated after sampling.

Air samples were collected with a small hand-held device consisting of a small chip disposed in a powerful electric field enabling to catch and retain bacteria and viruses from the air. When the device was switched on, it started to take in and filtrate air, which then passed by the small chip (Activity plan for air sampling technology, 2016). The air samples were collected the same day as the blood samples and from the same age groups (4, 8 and 12 weeks old pigs). Air samples were taken in two different ways (see Appendix 4). The first was called the "section measurement", where the operator of the device went slowly through the section with pigs spending a few minutes in each pen; the device should be kept as close as possible to the snouts of the pigs. The second was a "screening measure", where the operator went through the section quickly spending less time in each pen.

	0 week old	***4 weeks old***	***8 weeks old***	***12 weeks old***
PUCS	*X (12-16 samples)*			
Blood serum		*X (60 samples)*	*X (30 samples)*	*X (30 samples)*
Tonsil swabs	*X (60 samples)*			*X (30 samples)*
Air samples		*X*	*X*	*X*

Table 1. Sample collection from each herd. Blood samples and tonsil swabs from the 12 weeks old pigs were collected from the exact same pigs.

PUCS, blood serum and tonsil samples were marked with an unique identification number. After each sampling day, the samples were sent or transported to the National Veterinary Institute, Technical University of Denmark, where they were handled for further analysis.

The blood serum was centrifuged upon arrival (10 minutes at 3,000 rounds per minute (RPM) at a temperature of 5 °C). The serum was then collected and stored in serum tubes. Serum from pigs at 4 weeks of age from the same pen was pooled in 10 (100 µl serum from each serum sample pooled in an Eppendorf tube). Serum from pigs at 8 or 12 weeks of age from the same pen was pooled in 5 (200 µl serum from each serum sample pooled in an Eppendorf tube). The Eppendorf tubes were vortexed and centrifuged shortly, and 200 µl pooled sample was moved to a 2 ml Sarsted tube.

The PBS from the tonsil swabs was pooled in 5 (200 µl from each sample pooled in an Eppendorf tube). Here from, 200 µL was transferred to a 2 ml Sarsted tube.

Blood from the placentas was also centrifuged upon arrival (10 minutes at 3,000 RPM at 5 °C). The serum was collected and stored in serum tubes. These samples were not pooled. 200 µl was collected from the serum tubes and transferred to a 2 ml Sarsted tube.

All of the samples in the Sarsted tubes were frozen at -80 °C.

Regarding the air samples, the small chips were washed and then cleaned with a disinfectant wipe. 50 µl resubmission buffer was sucked into a pipette. An empty pipette was placed in the inner inlet and pressed down. The pipette with 50 µl resubmission buffer was placed in the outer inlet, and the fluid was moved back and forth between the two pipettes. After a while, the resubmission buffer was transferred to an Eppendorf tube. The resubmission buffer in the Eppendorf tube was then screened for PRRSV with real time RT-PCR. New gloves were used between each chip to avoid cross contamination. The authors of this thesis collected the air samples and screened the resubmission buffers for PRRSV with real time RT-PCR; Karsten Brandt Andersen, project manager at Point of Care, Force Technology, was responsible for the steps in-between.

3.5 RNA extraction and purification

Viral RNA was extracted from serum, PUCS and the PBS solution from the tonsil swabs. The pooled samples, which contained 100 µl, were mixed with 300 µl buffer. Viral RNA was extracted through automated purification on a QIAsymphony SP using QIAsymphony RNA kit 192 (all from

QIAGEN). RNA extraction was done in accordance to instructions from the manufacturer (protocol RNA 400 V7) with an elution volume of 100 µl. Each 23rd and 24th sample was a positive quality control (known PRRSV isolate) and a negative quality control (water), respectively. These procedures were also used for the preparation of samples to Sanger sequencing.

Extracted and purified RNA was stored at -80 °C (QIAsymphony handbook, 2016).

3.6 Real time RT-PCR

RNA obtained from the extraction/purification was tested for the presence of PRRSV using an assay developed and modified by Kleiboeker et al. (Kleiboeker Mod-1 real time RT-PCR assay) (Kleiboeker, S. B. et al., 2005; Wernike, K. et al., 2012). This assay is a multiplex and quantitative real time RT-PCR providing amplification of both PRRSV type 1 (EU) and type 2 (NA) using dual labeled chemistry. The structure of the primers and probes and their target regions are shown in table 2 (Kleiboeker, S. B. et al., 2005; Wernike, K. et al., 2012).

The real time RT-PCR used was a one step kit called QIAGEN OneStep RT-PCR kit (QIAGEN, OneStep RT-PCR, 2016) (see Appendix 5).

Primer	Orientation	Sequence structure 5'-3'	Target region
PRklm1-EU1fw	Genomic	GCACCACCTCACCCRRAC	ORF6,ORF7
PRklm1-EU2fw	Genomic	CAGATGCAGAYTGTGTTGCCT	
PRklm1-EU1rev	Reverse	CAGTTCCTGCRCCYTGAT	
PRklm1-EU2rev	Reverse	TGGAGDCCTGCAGCACTTTC	
PRklm1-NAfw	Genomic	ATRATGRGCTGGCATTC	ORF7,3'UTR
PRklm1-NArev	Reverse	ACACGGTCGCCCTAATTG	
PRklm1proEU1	Genomic	(6-FAM)-CCTCTGYYTGCAATCGATCCAGAC(BHQ1)	
PRklm1proEU2	Genomic	(6-FAM)-ATACATTCTGGCCCCTGCCCAYCACGT-(BHQ1)	
PRklm1proNA	Genomic	(TEX)-TGTGGTGAATGGCACTGATTGACA-(BHQ2)	

Table 2. Both primers and probes are in accordance to the Kleinboeker Mod-1 assay. EU: European (PRRSV type 1). NA: North American (PRRSV type 2). 6-FAM and TEX indicate the flourophores. BHQ1 and BHQ2 are the quenchers. (Source: Kleiboeker, S. B. et al., 2005; Wernike, K. et al., 2012).

3.7 Samples for sequencing

Of the total number of pooled samples, 9 blood serum pools and 4 tonsil swab pools (based on Ct-values) were selected for further analyses. All single/individual samples from these 13 pools were purified and tested with real time RT-PCR, and of all these single/individual samples, 14 blood serum single samples and 5 tonsil swab single samples were selected for Sanger sequencing based on Ct-values and age group. Of these 14 blood serum single samples, 10 were selected for NGS. The described selection process was done in cooperation with Lise Kirstine Kvisgaard (see Figure 14).

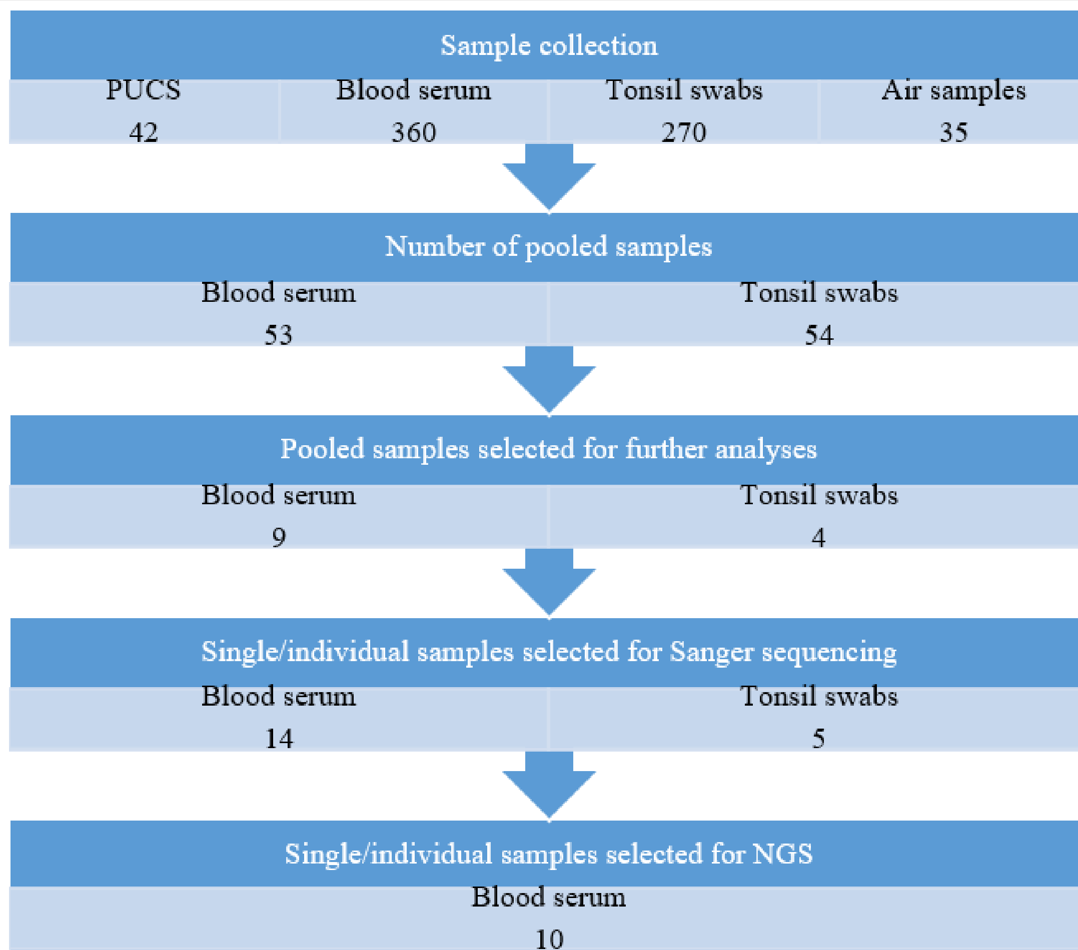


Figure 14. How samples were selected for sequencing. PUCS samples and air samples were never pooled but tested individually (single samples) from the beginning. None of the PUCS samples and air samples were chosen for sequencing analyses.

3.8 PCR of PRRSV type 2's ORF5 for Sanger sequencing

Since screening of our samples only showed presence of PRRSV type 2, only this type's ORF5-region was prepared for Sanger sequencing with QIAGEN OneStep RT-PCR kit (QIAGEN, OneStep RT-PCR, 2016) (see Appendix 6). Forward primer was 5'-GCTCCATTTCATGACACCTG-3', and reverse primer was 5'-AAAGGTGCAGAAG-CCCTAGC-3' (Oleksiewicz, M. B. et al., 1998).

After running the products on a gel, the products were purified (Purification of PCR products by Roche Applied Science, 2016). The isolates had their optic density (OD) measured using Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Scientific Nanodrop, 2008). The

corresponding computer program NanoDrop 1000 version 3.8.1 with the settings "Nucleo Acids" and "DNA 40" was used. The concentration of DNA was measured in ng/µl. The samples were sent to LGC Genomics GmbH (in Germany) for Sanger sequencing in a concentration of 200 ng with 5 µmol sequencing primer (see Appendix 7).

3.9 Preparation for NGS

RNA was extracted and purified from serum using Trizol LS, which is a ready-to-use agent used for isolation of high quality RNA (Trizol LS reagent, 2016). Equipment was cleaned with microsol before the procedure, and only tips from newly opened tip boxes were used. Furthermore, Eppendorf tubes were dry-sterilized before use. Gloves were worn during the entire procedure (see Appendix 8).

The procedure resulted in 30 eluted RNA samples, which were all tested by real time RT-PCR. The RNA concentration and quality were measured using Thermo Scientific NanoDrop 1000 spectrophotometer version 3.8.1 with the settings "Nucleo Acids" and "DNA 40". Concentration (ng/µl) and quality (good RNA quality: 260/280 ~ 2.0, 260/230 = 2.0-2.2) were measured (see Appendix 9). The 10 elutions with the lowest Ct-value were selected for further NGS analysis. RNA was kept at -80 °C until further use.

3.10 Preparation of RNA for NGS

Preparation of the extracted RNA from the Trizol purification for NGS was done with 2 different methods to compare their efficiency.

Method 1. Sequencing on PCR products. Full length cDNA synthesis was done with *SuperScript III first-Strand Synthesis System for RT-PCR (18080-051)* (SuperScript III first-Strand Synthesis System for RT-PCR, 2016), and long range PCR amplification for sequencing was made with *AccuPrime PCR amplification of PRRSV cDNA* (Taq DNA HF, 2016; Kvisgaard, L. K. et al., 2013b).

Full length cDNA synthesis took place in three continuous reactions. A RT primer was used (5'-CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT $T(T)_{38}$-3' (Nielsen, H. S. et al., 2003). All RNA was taken up to thaw. All reagents except enzymes were thawed, vortexed and

spun down. The enzymes were kept on ice until just before use and then spun down (see Appendix 10).

For long range PCR amplification, AccuPrime Taq DNA Polymerase High Fidelity was used (Taq DNA HF, 2016). All reagents except the polymerase were thawed at room temperature, vortexed and spun down before use. The polymerase was kept cool until use and was only spun down.

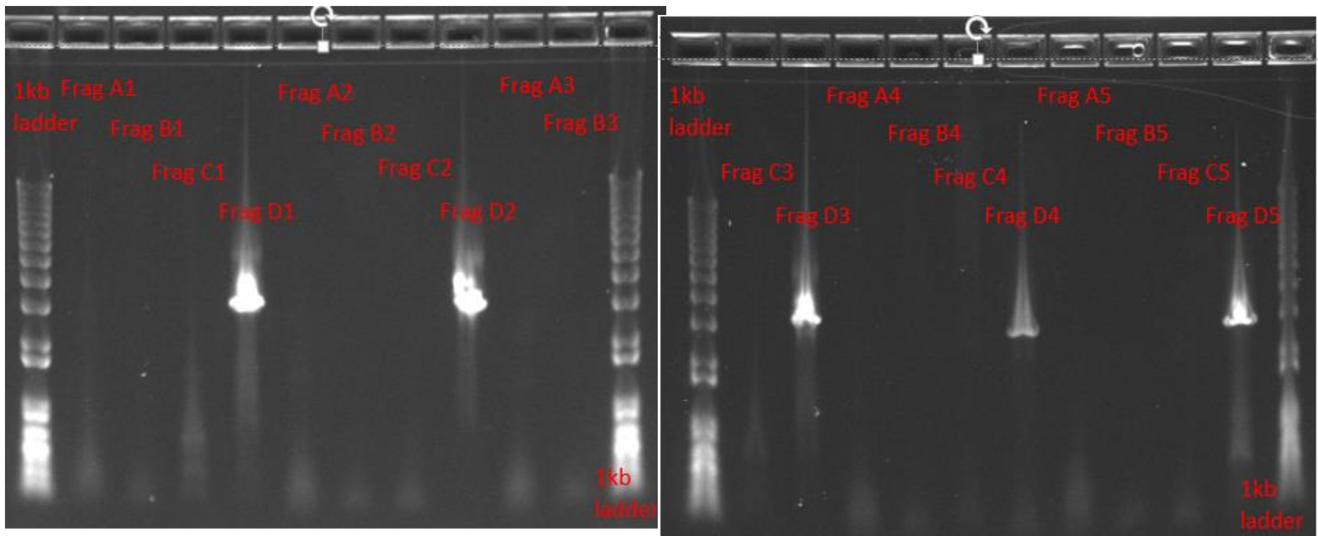
Four different PCR mixtures were made (see Appendix 11) using the following primers (Nielsen, H. S. et al., 2003; Fang, Y. et al., 2006; Darwich, L. et al., 2011; Diaz, I. et al., 2006):

- Fragment-A-US
    - Fragment-A-1-35-US-Fw: CTCGAGGGCGCGCCTAATACGACTCAC-TATAGGATGACGTATAGGTGTTGGCTCTATGCCTTGGCATT
    - Fragment-A-US-fw: GGAGGGCCAAGTCTACTGCACACGA
    - Fragment-A-US-Rev: GTGTCAGGGTCAACCACGA
    - Length: 4541/4811 bp
- Fragment-B-US
    - Fragment-B-US-Fw: ATGTTGGCTGGAGCTTACGT
    - Fragment-B-US-Rev: TGGTTGTGCTCAACCGCGT
    - Length: 3506 bp
- Fragment-C-US:
    - Fragment-C-US-Fw: TCTCAGAGTTGGCGACCCT
    - Fragment-C-US-Rev: ATGCTGCACCAAAGAGACCT
    - Length: 5500 bp
- Fragment-D-US:
    - Fragment-D-US-Fw: TTTCAGCATCTAGCCGCCA
    - Poly(dT)-RT: CAGGAAACAGCTATGACACCTGATCTCTAGAAACGTT(T)38
    - Length: 2900 bp

The PRRSV genome could also be covered using the following primers (Nielsen, H. S. et al., 2003; Fang, Y. et al., 2006; Darwich, L. et al., 2011; Diaz, I. et al., 2006):

- Fragment-1-US
    - Fragment-A-US-Fw: GGAGGGCCAAGTCTACTGCACACGA

   - Fragment-B-US-Rev: TGGTTGTGCTCAACCGCGT
   - Length: 7588 bp
- Fragment-2-US
   - Fragment-C-US-Fw: TCTCAGAGTTGGCGACCCT
   - Poly(dT)-RT: CAGGAAACAGCTATGACACCTGATCTCTAGAAACGTT(T)38
   - Length: 7939 bp



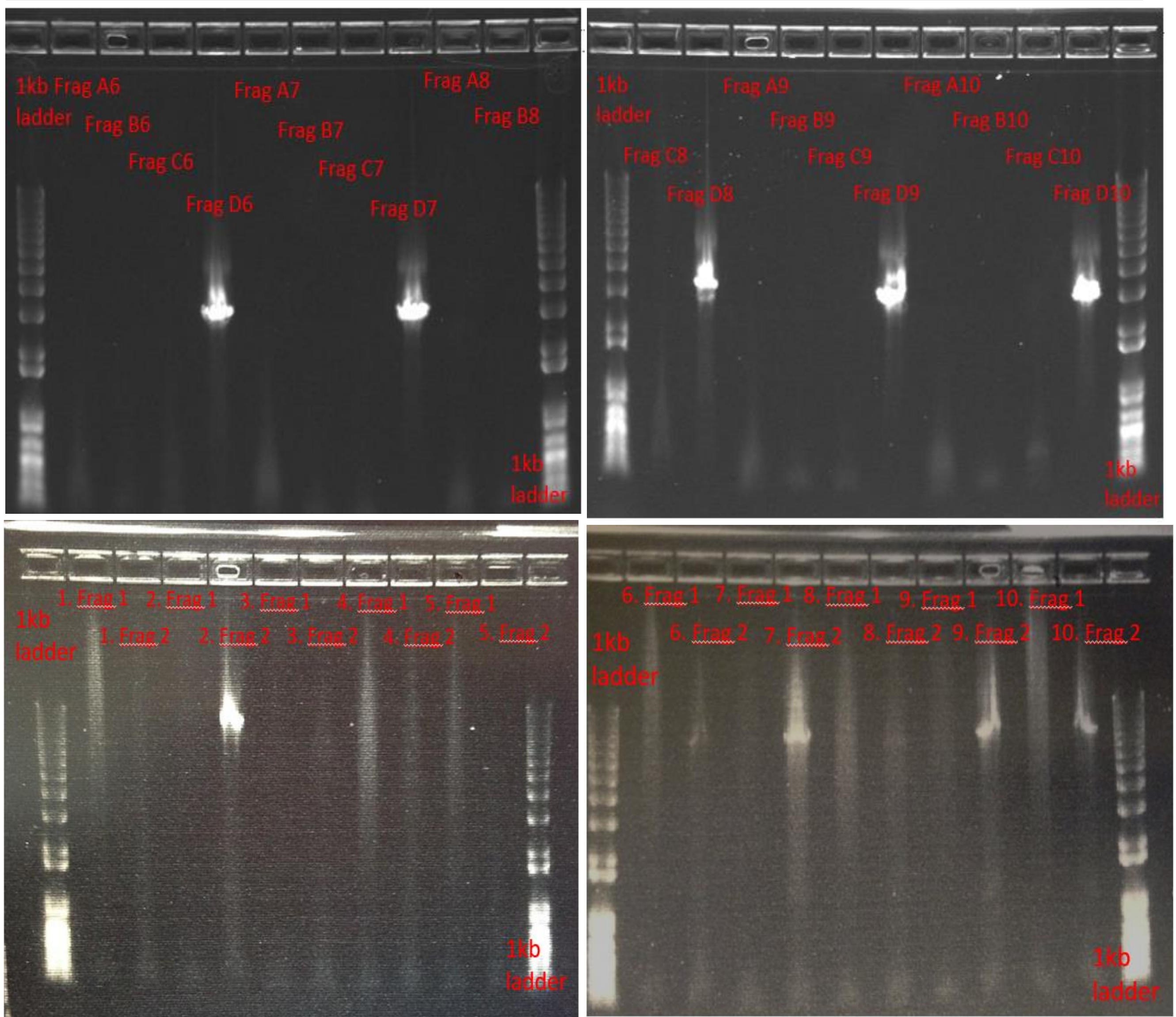


Figure 15. The top four pictures only show fragment D. The bottom two pictures with bands for fragment 2.

PCR products were run on an agarose gel electrophoresis using E-gel® 0.8% agarose gels from Invitrogen (10 µl, 1 kb plus ladder as reference and 5 µl PCR product) (see Figure 15). In the 10 samples tested, only fragment D appeared, so the procedure was repeated with new primers using fragment A as forward primer and fragment B as reverse primer ("Fragment 1"), and fragment C as forward primer and fragment RT-15392 as reverse primer ("Fragment 2"). The second PCR gel showed that sample 2, 7, 9 and 10 had bands for Fragment 2. Fragment 2 was then used for sequencing in these samples, fragment D was used in the other samples. The PCR products were puri-

fied (Purification of PCR products by Roche Applied Science, 2016). For each sample, the concentration was measured using Thermo Scientific NanoDrop 1000 spectrophotometer (program NanoDrop 1000 version 3.8.1 with the setting "Nucleo Acids" and "DNA 40"), and 1 μg was taken out and TE buffer added to an end volume of 130 μl (see Appendix 12 for samples 1-10). TE contains EDTA and Tris-buffer and stabilizes DNA (LifeTechnologies, 2015). The samples were sent to DTU and sequenced using Ion Torrent (carried out by senior researcher, chemist and molecular biologist Marlene Danner Dalgaard).

Method 2. Sequencing on double stranded DNA. Propagation of total RNA was done with the kit *QuantiTect Whole Transcriptome* from QIAGEN (QIAGEN Handbook, 2016), and synthesis of the second strand was done with *2nd strand synthesis of PRRSV* (DNA polymerase I, Large Fragment, NEbuffer 2, 2016).

The propagation takes place in three continuous reactions (QIAGEN Handbook, 2016). All enzymes were thawed on an ice block until just before use. All other reagents were thawed at room temperature. All buffers and reagents were vortexed and spun down before use (see Appendix 13).

Synthesis of the PRRSV second strand was done with DNA polymerase I, Large (Klenow) Fragment (M0210S), NEbuffer 2 (B7002S) (DNA polymerase I, Large Fragment, NEbuffer 2, 2016). All the reagents except the klenow fragment were thawed, vortexed and spun down. The klenow fragment was kept cool and spun down just before use. The procedure consists of 2 reactions (see Appendix 14).

The PCR products were purified (Purification of PCR products by Roche Applied Science, 2016). For each sample, the concentration was measured using Thermo Scientific NanoDrop 1000 spectrophotometer (program NanoDrop 1000 version 3.8.1 with the setting "Nucleo Acids" and "DNA 40"), and 1 μg was taken out and TE buffer added to an end volume of 130 μl (see Appendix 12 for samples 11-22). TE contains EDTA and Tris-buffer and stabilizes DNA (LifeTechnologies, 2015). The samples were sent to DTU and sequenced using Ion Torrent (carried out by senior researcher, chemist and molecular biologist Marlene Danner Dalgaard).

3.11 Analysis of sequencing data

Sanger sequencing. The analysis of the 18 sequenced ORF5 segments was done using the computer program CLC Main Workbench version 7.0. The sequences were imported into the program, and

every sequence (forward and reverse) for every sample was compared to a reference (EF484033.1) using "toolbox" and "assemble sequences to reference". Hereby, the sequences were analysed, and a consensus sequence was obtained. An alignment was then made with the consensus sequences from each herd and with all the consensus sequences using "toolbox" and "create alignment" to see the differences within the herd and the differences between the herds. Furthermore, a pairwise comparison was made (including comparison with the Ingelvac PRRS® MLV vaccine) to examine for differences. A phylogenetic tree was constructed with our sequences as well as 79 reference sequences (delivered by Lise Kirstine Kvisgaard) using the program FigTree version 1.4.3.

Using the function "translate to protein", an amino acid sequence was obtained. An amino acid analysis of the ORF5's GP5 was carried out with the focus on the putative N-glycosylation site (aa34), the decoy epitope motif (aa27-30), the cysteine residue (aa48) and the neutralizing epitope (aa37-45) (Ostrowski, M. et al., 2002; Mardassi, H. et al., 1996).

NGS Ion Torrent. The 10 samples chosen for NGS were prepared in two ways: 10 samples with single fragments (2.8 kb and 7.9 kb), and 10 samples with a mix of unspecific primers. At DTU, the fragments were cut into equal sizes (400 bp), and since all the samples were poled in the sequencing process, a barcode sequence was added to each sample. This barcode was later removed and was not a part of the sequence data (Ion Torrent Amplicon Sequencing, 2011). The sequence data from DTU was imported into the "Ion Torrent Import"-function in the program CLC Genomics Workbench version 6.5. The reads were trimmed using the toolbox "Trim Sequence" and default settings were used. The total number of reads from each sample was assembled in two different ways. Firstly, the reads were assembled using Ingelvac PRRS® MLV vaccine as a reference sequence ("Map Reads to Reference"-function with default settings) creating a "consensus assembly". Hereafter, the reads were assembled using the "De Novo Assembly"-function with default settings and the setting "Create Report" under "Result Handling" to create an assembly summary report. The de novo sequence and its direction were identified using NCBI's BLAST. When looking for variations on gene level (quasispecies), raw data of each sample was mapped against its own consensus sequence (both Ingelvac PRRS® MLV vaccine as reference and the de novo) using the "Quality-based Variant Detection"-function. Here, all the reads were compared to find differences between them. The variants found could be single nucleotide variants (SNVs), multiple nucleotide variants (MNVs), replacements, deletions or insertions. When searching for a variant, the program

also takes into account the quality of the neighbouring bases, and thereby, it estimates whether the variant is probable in relation to the specific area of the genome (CLC-Workbench, 2016). Due to the late arrival of NGS Ion Torrent data from DTU, the data was processed in CLC Genomics Workbench by Lise Kirstine Kvisgaard.

4 Statistical analyses

The collected data was analysed using Microsoft Office Excel. Student's t-test was used to compare Ct-values between blood serum and tonsil swabs (from 12 weeks old pigs from all three herds overall) as well as Ct-values from blood serum between different age groups. In order to use this test, the data has to be normally distributed. In addition, the two populations have to have the same variance (tested with a F-test, similar variance if p-value is over 0.05). These analyses were made on pooled samples and not individual samples. To compare data from the clinical registrations (symptoms) with PRRSV status in blood serum, Fisher's exact test was used, since the number of counts in each category was lower than five (Statistical notes, 2016).

A two-sided p-value lower than 0.05 was considered statistically significant (rejecting our hypotheses H_0 of no differences between compared groups).

Results

1 Management related observational study

1.1 Questionnaire

All three herds responded to the questionnaire (see Appendix 1), and only very few questions were left unanswered. If some of the questions lacked detailed explanations or were unanswered, a new e-mail was sent or the herd was contacted by phone.

1.1.1 Productivity data

Productivity data is available in Appendix 15. It includes clinically relevant (for PRRSV) parameters from the examined herds compared with the national average from 2015 (Jessen, O., 2016). Data from both farrowing and nursery units is included.

Herd 1 (see Table 3). All parameters are better than the national average except "weight at weaning", which is 2.1 kg lower. In addition, herd 1 has a daily gain of 467 g (national average: 444 g).

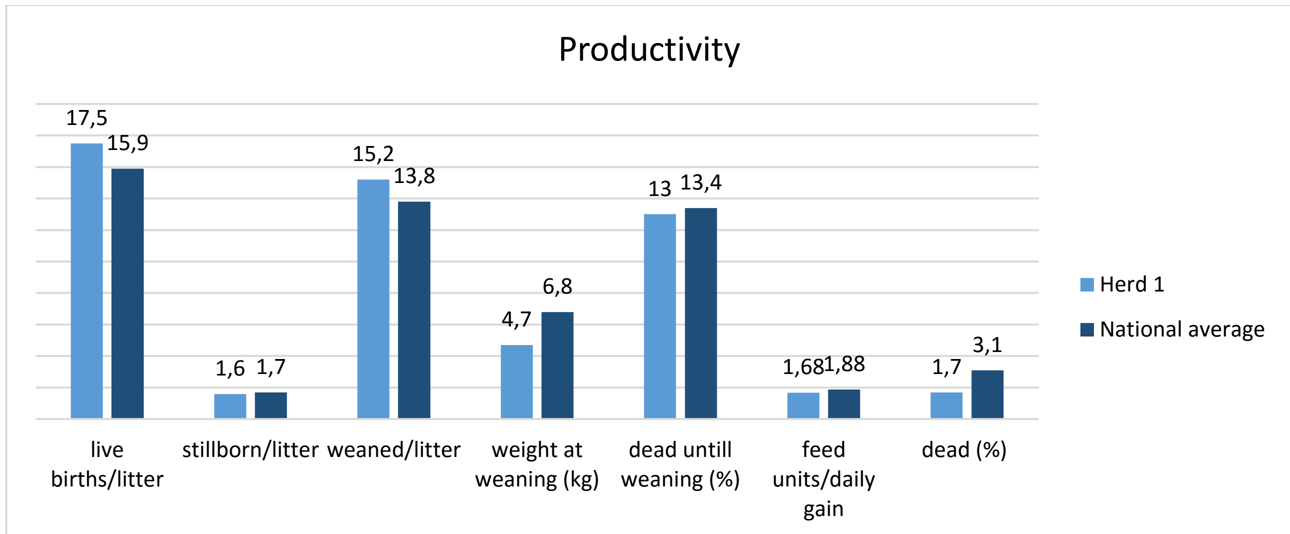


Table 3. Productivity data from herd 1 compared with national average from 2015.

Herd 2 (see Table 4). Almost all parameters are better than the national average. However, weight at weaning is 1.38 kg lower than the average, and dead until weaning is 1.16% higher than the average. Herd 2 has a daily gain of 448 g (national average: 444 g).

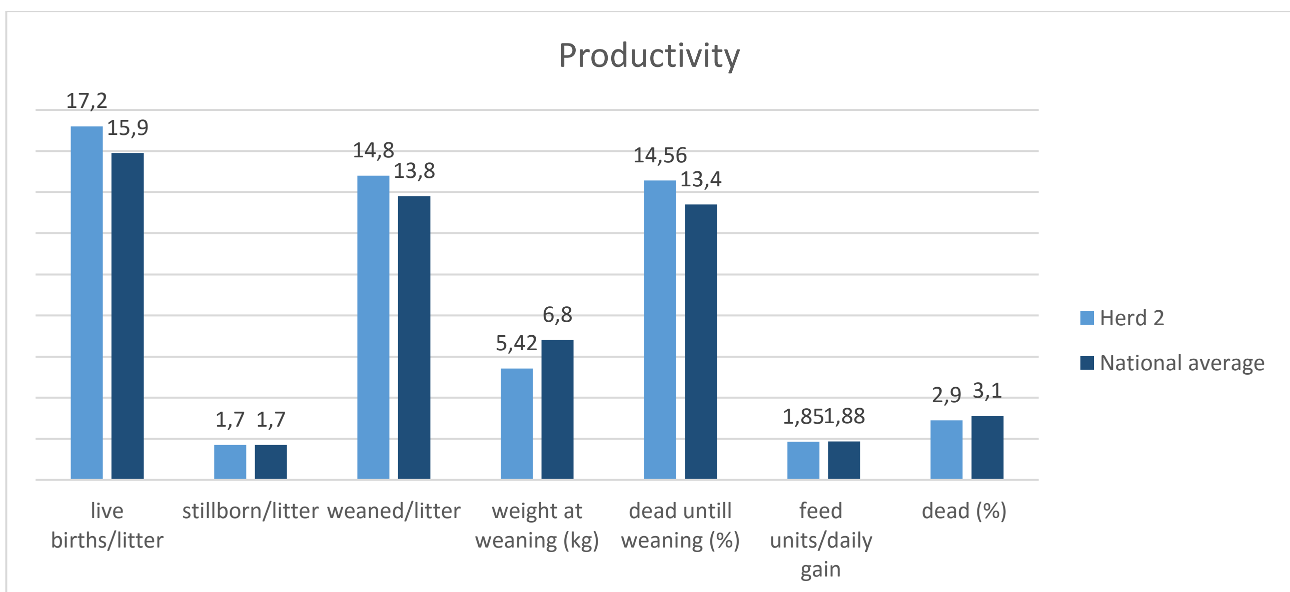


Table 4. Productivity data from herd 2 compared with the national average.

Herd 3 (see Table 5). Stillborn/litter is 0.2 higher, the herd weans 0.2 piglets less, and dead until weaning is 6.5% higher than the national average. Herd 3 has a daily gain of 408 g (national average: 444 g).

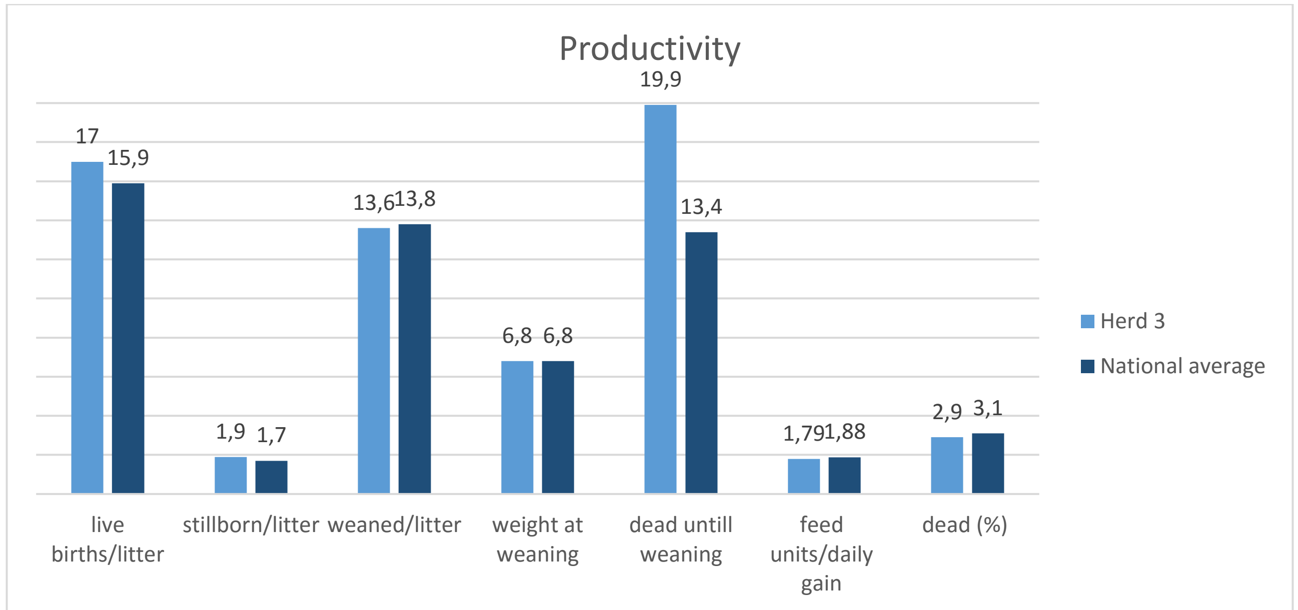


Table 5. Productivity data from herd 3 compared with the national average.

1.1.2 Antibiotic use

Table 6 and Figure 16 show the ADD per 100 animals per day as an average for the last 9 months (registered in Vetstat) for all three herds compared with national limit/threshold values (see Appendix 16). None of the herds had ADD values over the national limit/threshold value.

	Herd 1	Herd 2	Herd 3	National limit/threshold value
ADD (piglets/sows)	1.19	1.64	2.69	4.3
ADD (nursery)	12.43	13.34	11.11	22.9

Table 6. ADD values for all three herds.

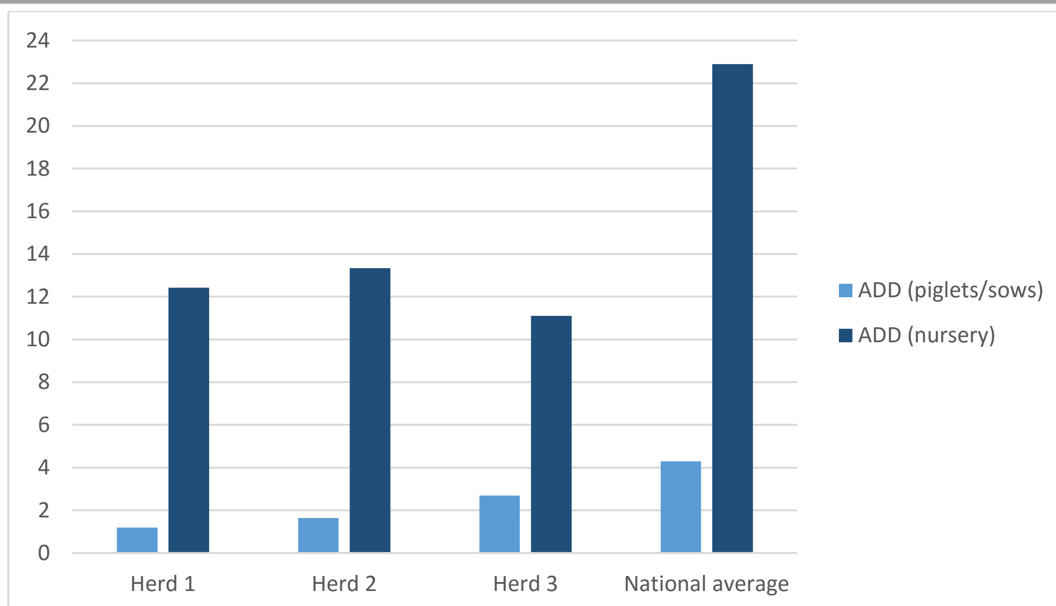


Figure 16. ADD according to piglets/sows and nursery in the three herds.

1.1.3 Management

An overview of the three herds' management strategies and procedures is shown in Table 7. Comparison of these and the guidelines (McRebel) shows that none of the herds follows McRebel completely. In addition, we found that the three herds vary in which parts of McRebel they follow.

	Herd 1	***Herd 2***	***Herd 3***
Clinical signs of PRRS	No	Yes	Yes, unthrifty pigs in nursery section
Floor heating in piglet corner	Yes	No, but heating lampes	Yes
Cross-fostering before 24 hours after farrowing	No	Yes	Start: Earlier than 24 hours End: 3 days
Nurse sows	Yes	Yes	Yes
If early weaning (nurser sows), do piglets move to nursery section	Yes	Yes	No, stays in the farrowing section
Sectioned farrowing teams - AI/AO principles	No	Yes	Yes
AI/AO principles in nursery section	Yes	No, not always	No, not always

Small and weak piglets at weaning	Foster sow in a different section (continuous flow)	Euthanize if the piglet is weak, but a whole litter can be moved to buffer unit	Euthanize if the piglet is weak
Wash of farrowing section	Yes, without soap	Yes, with soap	Yes, with soap
Drying of farrowing section	Air and heat canon	Air (summer) Heat canon (winter)	Air
Time for drying	Min. 12 hours	2 days	1-2 days
Disinfection of farrowing section	Yes	Yes	Yes
Wash of nursery section	Yes, without soap	Yes, with soap	Yes, with soap
Drying of nursery section	Air	Air (summer) Heat canon (winter)	Air and heat canon
Time for drying	Min. 24 hours	2 days	1-3 days
Disinfection of nursery section	Yes	Yes	Yes
Wash of boots/hands	Never	After each unit	Never
Change of needle	Between each section	Between each section	Between each section
Tail docking (cleaning)	Between each piglet	Between each litter	Between each litter
How to clean instrument for castration	In water and alcohol	Chlorhexidin alcohol	In alcohol
When to clean instrument for castration	Between each piglet	Between each piglet	Between each piglet

Table 7. Summary of questionnaire for all three herds.

Table 7 shows that all of the 3 herds use nurse sows. When a nurse sow is made, herd 1 and 2 move the piglets from this sow to nursery section, but herd 3 leaves them in farrowing unit until the day of weaning. Only herd 2 uses cross-fostering earlier than 24 hours after farrowing. Herd 3 starts to cross-foster earlier than 24 hours but does not finish it before 3 days after. At weaning, the smallest and weakest piglets are euthanized in herd 2 and 3; in herd 1, they move them to a foster sow in another section. Herd 1 cannot follow AI/AO principles in farrowing unit due to the high numbers of sows and the fact that they produce 36.7 weaned piglets/year sow (see Appendix 5). However, herd

1 follows AI/AO principles in nursery unit, which herd 2 and 3 do not due to lack of space. All herds wash and use disinfection but vary in how to dry and the period of time used for drying. Only herd 2 washes boots and hands between units.

1.2 Clinical registration (symptoms)

Results from clinical registration (see Appendix 17) are shown in Table 8 illustrated as prevalences of symptoms (unthrifty pigs, respiratory distress and blobs of diarrhea) according to age groups.

	Age (weeks)	**Unthrifty (prevalence in %)**	**Respiratory distress (prevalence in %)**	**Diarrheal blobs (no. blobs/ no. pens)**
Herd 1	4	1.7	3.4	4/18
	8	0.0	1.7	2/18
	12	0.0	0.8	5/18
Herd 2	4	0.0	1.7	0/8
	8	1.3	4.5	0/8
	12	0.9	4.5	0/8
Herd 3	4	2.8	90-100 (sneezing) 1.2 (coughing)	3/12
	8	1.9	2.1	0/12
	12	0.0	1.4	0/12

Table 8. The results of the clinical registration.

Herd 1 had the largest number of diarrheal blobs observed, but it did not reach an average of 1-3 blobs of diarrhea in the different pens, which is usually required before initiating antibiotic treatment (Pedersen. K. S, 2014). Herd 3 had the largest number of unthrifty pigs and a high prevalence of sneezing in 4 weeks pigs (90-100%). However, respiratory symptoms could not be observed in the older age groups (in nursery unit). Herd 2 had the highest number of pigs with respiratory dis-

tress. Importantly, none of the three herds had high occurrence of symptoms from the three categories (unthrifty pigs, respiratory distress, blobs of diarrhea).

2 Viral detection and genetic characterization

2.1 Real time RT-PCR

The real time RT-PCR results from the pooled samples (and for PUCS, individual samples) are summarized in Table 9 (herd 1), Table 10 (herd 2) and Table 11 (herd 3). Results are shown as number of pooled samples positive for PRRSV out of the total number of pooled samples tested. In addition, these tables include a mean Ct-value. Ct-values for all pooled samples can be seen in Appendix 18.

	0 weeks old	***4 weeks old***	***8 weeks old***	***12 weeks old***
PUCS	*0/14*			
Blood serum		*0/6*	*0/6*	*5/6 Mean Ct-value: 30.78 [27.53-33.64]*
Tonsil swabs	*0/12*			*5/6 Mean Ct-value: 35.85 [33.65-37.49]*
Air samples	*0*	*0/3*	*0/3*	*0/2*

Table 9. Real time RT-PCR results from herd 1.

	0 weeks old	***4 weeks old***	***8 weeks old***	***12 weeks old***
PUCS	*0/12*			
Blood serum		*0/6*	*4/5 Mean Ct-value: 32.14 [26.79-36.12]*	*6/6 Mean Ct-value: 32.3 [27.55-36.85]*
Tonsil swabs	*0/12*			*6/6 Mean Ct-value: 36.49 [35.24-38.28]*
Air samples	*0*	*0/3*	*0/3*	*0/9*

Table 10. Real time RT-PCR results from herd 2.

	0 weeks old	***4 weeks old***	***8 weeks old***	***12 weeks old***
PUCS	*0/16*			
Blood serum		*3/6* *Mean Ct-value: 35.33* *[31.45-37.65]*	*2/6* *Mean Ct-value: 34.32* *[33.31-35.32]*	*6/6* *Mean Ct-value: 31.71* *[25.62-38.46]*
Tonsil swabs	*0/12*			*6/6* *Mean Ct-value: 33.14* *[31.27-37.14]*
Air samples	*0*	*0/3*	*0/3*	*0/6*

Table 11. Real time RT-PCR results from herd 3.

A brief overview of the 13 pooled samples (tested positive for PRRSV) from which all individual/single samples were further analysed is given in Appendix 19. The real time RT-PCR results of the individual/single samples tested positive for PRRSV are shown in Appendix 20. Real time RT-PCR results of the PRRSV positive individual/single samples chosen for Sanger sequencing and/or NGS are shown in Appendix 21. Results from the ORF5 PCR (preparation for Sanger sequencing) are shown in Appendix 22. Results from the gel electrophoresis on ORF5 PCR products are shown in Figure 17. As seen, one sample (12:197(b3-s42)) did not show a band, even after a second attempt, and this sample was therefore excluded. Concentrations of the samples sent to Sanger sequencing are shown in Appendix 7.

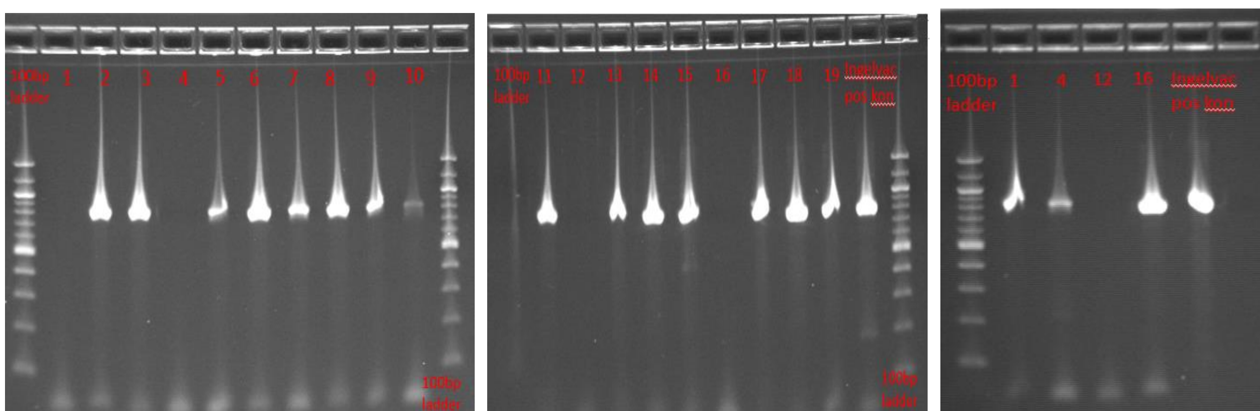


Figure 17. Gel electrophoresis results from the PRRSV type 2 ORF5 PCR. Picture to the right shows the results from the second attempt.

2.2 Sanger sequencing of PRRSV type 2's ORF5

Of the 36 samples (a forward and reverse strand for each of the 18 single/individual samples), 35 came back with useful sequences (sample 191 reverse (B2-t111) did not provide any useful sequence). The consensus sequences obtained were aligned with relevant type 2 reference strands (see Appendix 23). A phylogenetic tree was created (see Figure 18). Pairwise comparison between the obtained sequences and the Ingelvac PRRS® MLV is visualised in Figure 18.

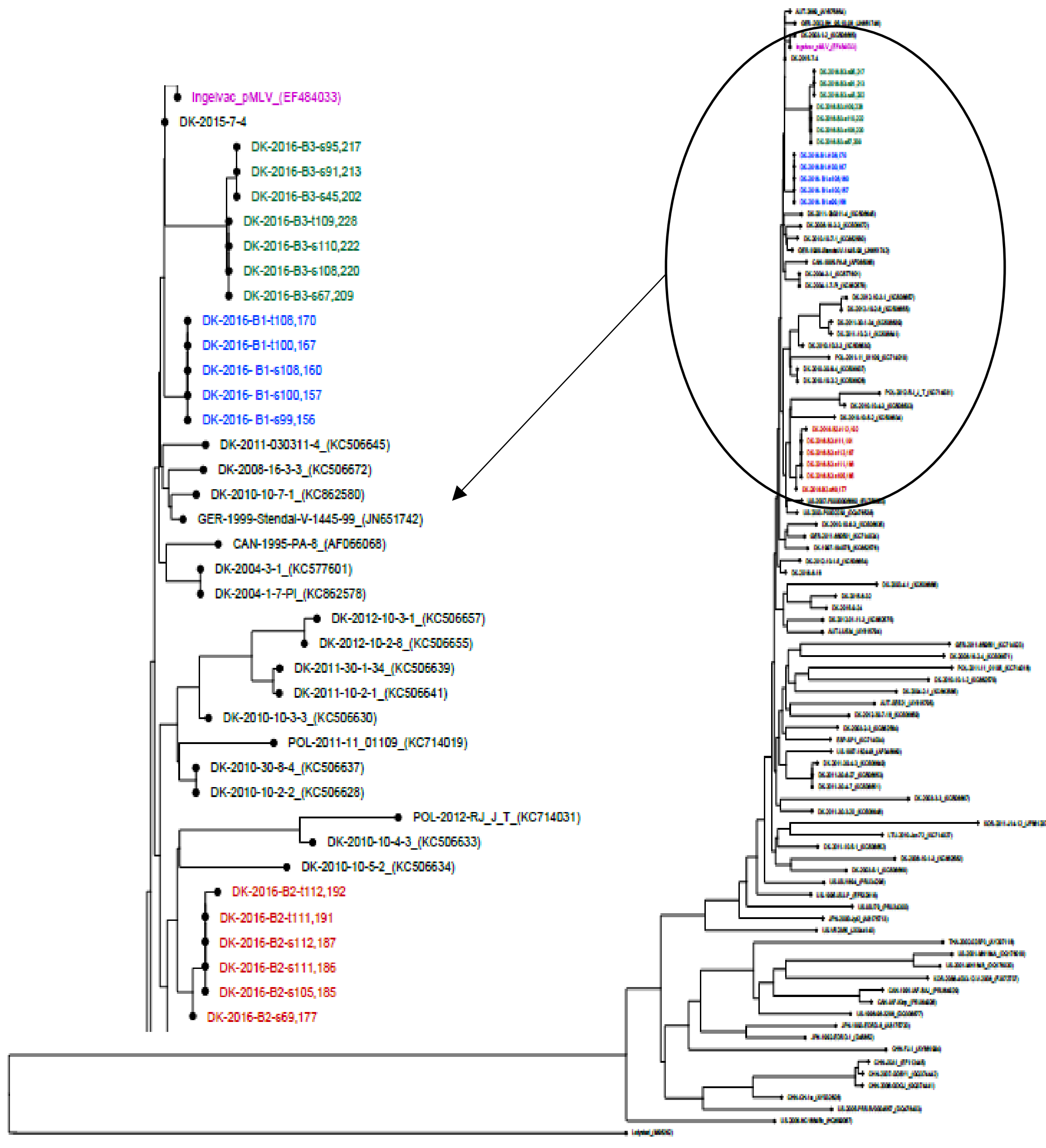


Figure 18. Phylogenetic tree based on our obtained ORF5 sequences and sequences delivered by Lise K. Kvisgaard.

The sequences from herd 1 were all 99.50% similar to Ingelvac PRRS® MLV with only three mutations in difference (see Figure 18). The sequences from herd 2 were between 98.84-99.17% similar to Ingelvac PRRS® MLV with 5-7 mutations in difference (see Figure 19). The sequences from herd 3 were between 98.84-99.00% similar to Ingelvac PRRS® MLV vaccine (see Figure 19). The phylogenetic analysis showed that all sequences were placed in one lineage (lineage 5, sublineage 5.1), which contains strains closely related to Ingelvac PRRS® MLV and ATCC VR-2332 (Shi, M. et al., 2010a). Furthermore, the phylogenetic tree shows that the sequences cluster together on herd level (see Figure 18).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Ingelvac_pMLV_(EF484033)	1		3	3	3	3	3	5	6	6	6	6	7	7	6	7	7	6	6	6
DK-2016- B1-s99,156	2	99,50		0	0	0	0	6	7	7	7	7	8	8	7	8	8	7	7	7
DK-2016- B1-s100,157	3	99,50	100,00		0	0	0	6	7	7	7	7	8	8	7	8	8	7	7	7
DK-2016- B1-s108,160	4	99,50	100,00	100,00		0	0	6	7	7	7	7	8	8	7	8	8	7	7	7
DK-2016-B1-t100,167	5	99,50	100,00	100,00	100,00		0	6	7	7	7	7	8	8	7	8	8	7	7	7
DK-2016-B1-t108,170	6	99,50	100,00	100,00	100,00	100,00		6	7	7	7	7	8	8	7	8	8	7	7	7
DK-2016-B2-s69,177	7	99,17	99,00	99,00	99,00	99,00	99,00		1	1	1	1	2	10	9	10	10	9	9	9
DK-2016-B2-s105,185	8	99,00	98,84	98,84	98,84	98,84	98,84	99,83		0	0	0	1	11	10	11	11	10	10	10
DK-2016-B2-s111,186	9	99,00	98,84	98,84	98,84	98,84	98,84	99,83	100,00		0	0	1	11	10	11	11	10	10	10
DK-2016-B2-s112,187	10	99,00	98,84	98,84	98,84	98,84	98,84	99,83	100,00	100,00		0	1	11	10	11	11	10	10	10
DK-2016-B2-t111,191	11	99,00	98,84	98,84	98,84	98,84	98,84	99,83	100,00	100,00	100,00		1	11	10	11	11	10	10	10
DK-2016-B2-t112,192	12	98,84	98,67	98,67	98,67	98,67	98,67	99,67	99,83	99,83	99,83	99,83		12	11	12	12	11	11	11
DK-2016-B3-s45,202	13	98,84	98,67	98,67	98,67	98,67	98,67	98,34	98,18	98,18	98,18	98,18	98,01		1	0	0	1	1	1
DK-2016-B3-s67,209	14	99,00	98,84	98,84	98,84	98,84	98,84	98,51	98,34	98,34	98,34	98,34	98,18	99,83		1	1	0	0	0
DK-2016-B3-s91,213	15	98,84	98,67	98,67	98,67	98,67	98,67	98,34	98,18	98,18	98,18	98,18	98,01	100,00	99,83		0	1	1	1
DK-2016-B3-s95,217	16	98,84	98,67	98,67	98,67	98,67	98,67	98,34	98,18	98,18	98,18	98,18	98,01	100,00	99,83	100,00		1	1	1
DK-2016-B3-s108,220	17	99,00	98,84	98,84	98,84	98,84	98,84	98,51	98,34	98,34	98,34	98,34	98,18	99,83	100,00	99,83	99,83		0	0
DK-2016-B3-s110,222	18	99,00	98,84	98,84	98,84	98,84	98,84	98,51	98,34	98,34	98,34	98,34	98,18	99,83	100,00	99,83	99,83	100,00		0
DK-2016-B3-t109,228	19	99,00	98,84	98,84	98,84	98,84	98,84	98,51	98,34	98,34	98,34	98,34	98,18	99,83	100,00	99,83	99,83	100,00	100,00	

Figure 19. Pairwise comparison of our obtained ORF5 sequences and Ingelvac PRRS® MLV.

Examining the ORF5 amino acid sequences (see Figure 20), several positions in relation to GP5 were screened for relevant changes. The putative N-glycosylation site at aa34 was conserved in all three herds (Mardassi, H. et al., 1996). The aa27-30 (A/VLA/VN motif), which is considered to contain the decoy epitope, was conserved in all three herds. The cysteine residue at aa48 and the neutralizing epitope at position aa37-45 (SHL/QLIYNL motif) were also conserved in all three herds (Ostrowski, M. et al., 2002).

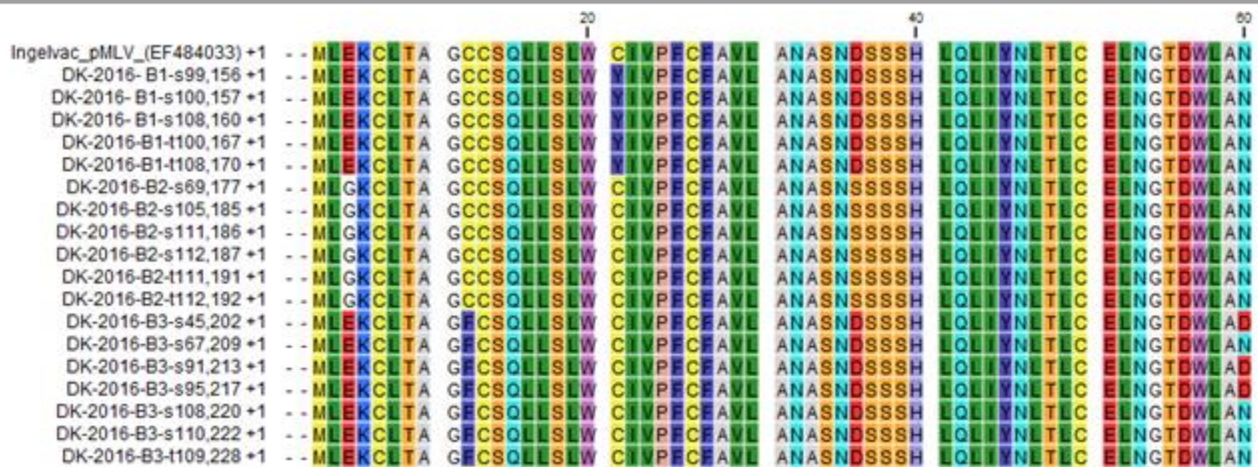


Figure 20. Amino acid sequences (aa1-60) from all three herds and Ingelvac PRRS® MLV.

2.3 Next-Generation Sequencing

A brief overview of the 20 samples sent to NGS Ion Torrent is given in Appendix 13. Samples 15-24 were prepared using method 1, samples 25-34 were prepared using method 2. Samples 25-34 were not of very god quality and the results from these are therefore not addressed further. It was only possible to obtain parts of the genome in the propagation process, so only parts of the genome were sequenced.

2.3.1 Next-Generation Sequencing results

Sample 15(15). After trimming, the total number of reads was 15,535 with an average length of 146.5. Using “de novo assembly”, a consensus length of 2,900 bases was obtained and average coverage was 720.55. The coverage determines how many times a single base at a certain position has been sequenced. A debt of maximum 1,293 was reached. A total of 28 contigs was obtained with an average length of 468 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to *Porcine respiratory and reproductive syndrome virus strain PL97-1/Lp1, complete genome*. The results of the “variant detection” showed 13 variants. These were all single nucleotide deletions.

Sample 16(16). After trimming, the total number of reads was 19,249 with an average length of 150.4. Using “de novo assembly”, a consensus length of 8,012 bases was obtained and average coverage was 345.08. A debt of maximum 619 was reached. A total of 15 contigs were obtained with an average length of 917 bases. A consensus BLAST was executed in NCBI, and the sample

showed to be 99% similar to *Porcine reproductive and respiratory syndrome virus isolate MLV RespPRRS/Repro, complete genome.* The results of the "variant detection" showed 36 variants. Of these, 35 were single nucleotide deletions and one was a single nucleotide variation.

Sample 17(21). After trimming, the total number of reads was 23,125 with an average length of 148.8. Using "de novo assembly", a consensus length of 2,946 bases was obtained and average coverage was 1,120.85. A debt of maximum 1,577 was reached. A total of 14 contigs were obtained with an average length of 688 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine respiratory and reproductive syndrome virus strain rV63, complete genome.* The results of the "variant detection" showed 14 variants. These were all single nucleotide deletions.

Sample 18(42). After trimming, the total number reads was 6,143 with an average length of 144.5. Using "de novo assembly", a consensus length of 2,909 bases was obtained and average coverage was 289.81. A debt of maximum 468 was reached. A total of 5 contigs were obtained with an average length of 1,004 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine respiratory and reproductive syndrome virus strain rV63, complete genome*. The results of the "variant detection" showed 14 variants. These were all single nucleotide deletions.

Sample 19(23). After trimming, the total number of reads was 37,856 with an average length of 142. Using "de novo assembly", a consensus length of 2,911 bases was obtained and average coverage was 1,454.57. A debt of maximum 2,725 was reached. A total of 30 contigs were obtained with an average length of 653 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine respiratory and reproductive syndrome virus strain rV63, complete genome.* The results of the "variant detection" showed 13 variants. These were all single nucleotide deletions.

Sample 20(25). After trimming, the total number of reads was 8,422 with an average length of 147.2. Using "de novo assembly", a consensus length of 2,910 bases was obtained and average coverage was 413.7. A debt of maximum 666 was reached. A total of 8 contigs were obtained with an average length of 690 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine respiratory and reproductive syndrome virus strain rV63,*

complete genome. The results of the "variant detection" showed 10 variants. These were all single nucleotide deletions.

Sample 21(26). After trimming, the total number of reads was 15,379 with an average length of 143.4. Using "de novo assembly", a consensus length of 5,251 bases was obtained and average coverage was 251.81. A debt of maximum 433 was reached. A total of 17 contigs were obtained with an average length of 838 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine reproductive and respiratory syndrome virus isolate HK14, complete genome.* The results of the "variant detection" showed 40 variants. 39 of these were single nucleotide deletions, and one single nucleotide variant was found.

Sample 22(27). After trimming, the total number of reads was 11,837 with an average length of 147.7. Using "de novo assembly", a consensus length of 2,909 bases was obtained and average coverage was 576.05. A debt of maximum 955 was reached. A total of 12 contigs were obtained with an average length of 641 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine respiratory and reproductive syndrome virus strain PL97-1/LP1, complete genome.* The results of the "variant detection" showed 13 variants. These were all single nucleotide deletions.

Sample 23(28). After trimming, the total number of reads was 19,919 with an average length of 141.5. Using "de novo assembly", a consensus length of 6,928 bases was obtained and average coverage was 330.25. A debt of maximum 602 was reached. A total of 13 contigs were obtained with an average length of 991 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine respiratory and reproductive syndrome virus strain Clone20, complete genome.* The results of the "variant detection" showed 43 variants. 41 of these were single nucleotide deletions, one single nucleotide variant was found, and one two-nucleotide deletion was found.

Sample 24(29). After trimming, the total number of reads was 20,555 with an average length of 133.8. Using "de novo assembly", a consensus length of 7,368 bases was obtained and average coverage was 328.48. A debt of maximum 551 was reached. A total of 17 contigs were obtained with an average length of 868 bases. A consensus BLAST was executed in NCBI, and the sample showed to be 99% similar to the *Porcine reproductive and respiratory syndrome virus iso-*

late HK14, complete genome. The results of the "variant detection" showed 39 variants. 38 of these variants were single nucleotide deletions, and one was a two-nucleotide deletion.

To summarize, the results from NGS Ion Torrent sequencing showed very little variation in the samples and only in form of single nucleotide deletions and a few single nucleotide variations. Thereby, no different quasispecies were found. Comparing the NGS consensus sequences (both reference and de novo) with the corresponding ORF5 Sanger sequences, we found that they were 100% identical.

3 Hypotheses

3.1 H_0 number 1: There is no significant difference in PRRSV occurrence when comparing real time RT-PCR results from PUCS and tonsil swabs from 0 weeks old pigs.

None of the collected PUCS samples or tonsil swabs from any of the 3 herds was positive for PRRSV. Thereby, no comparison can be made on a statistical level. Based on our findings, no difference in PRRSV occurrence between PUCS samples and tonsil swabs from 0 weeks old pigs could be found.

3.2 H_0 number 2: There is no significant difference in PRRSV occurrence when comparing real time RT-PCR results from blood serum and tonsil swabs from 12 weeks old pigs.

Pooled blood serum samples tested PRRSV positive corresponded to the same pooled tonsil swab samples tested PRRSV positive (see Table 12).

HERD 1

Sample number	Blood serum (Ct-value)	Tonsil swab (Ct-value)
13	33.64	35.86
14	30.97	33.65
15	31.45	36.37
16	27.53	35.88
18	30.29	37.49

HERD 2

Sample number	Blood serum (Ct-value)	Tonsil swab (Ct-value)
13	34.4	35.7
14	36.85	37.01
15	29.36	36.45
16	31.1	38.28
17	27.55	36.23
18	34.55	35.24

HERD 3

Sample number	Blood serum (Ct-value)	Tonsil swab (Ct-value)
13	26.57	33.31
14	32.06	32.45
15	30.15	32.54
16	25.62	31.27
17	38.46	37.14
18	37.4	32.15

Table 12. Comparison of pooled blood serum samples and pooled tonsil swab samples (Ct-values) according to age group from all three herds.

No difference in PRRSV occurrence (positive versus negative) when comparing pooled blood serum samples and pooled tonsil swab samples from 12 weeks old pigs was found. However, using a Student's t-test, Ct-values from blood serum samples were significantly lower ($p = 0.003$) than Ct-values from tonsil swab samples.

3.3 H_0 number 3: There is no significant difference between virus load (Ct-values) in the different age groups.

In herd 1, PRRSV was only found in 12 weeks old pigs. In herd 2 and 3, PRRSV was found in 8 and 12 weeks old pigs. In addition, in herd 3, PRRSV was also found in 4 weeks old pigs. Statistical

analyses (Student's t-test) on the difference in Ct-values between the different age groups showed the following results:

Herd 2:	Serum from 8 and 12 weeks old pigs	**p = 0.95**
Herd 3:	Serum from 8 and 12 weeks old pigs	**p = 0.54**
	Serum from 4 and 12 weeks old pigs	**p = 0.33**
	Serum from 4 and 8 weeks old pigs	**p = 0.72**

As seen, no statistically significant difference between the age groups' Ct-values (virus load) was found.

3.4 H_0 number 4: There is no significant difference between PRRSV quasispecies and the age of the pigs.

Of the 10 samples selected for NGS Ion Torrent, 2 came from herd 1 (12 weeks old pigs), 4 came from herd 2 (1 from 8 weeks old pigs, 3 from 12 weeks old pigs), and 4 came from herd 3 (1 from 4 weeks old pigs, 1 from 8 weeks old pigs, 2 from 12 weeks old pigs). Based on our obtained sequences, no presence of quasispecies was found in any of the samples. The sample size, the sample distribution according to age groups and the lack of full genome possibly made it insufficient to illustrate the presence of different quasispecies between the different age groups.

3.5 H_0 number 5: There is no significant difference between signs of clinical illness and PRRSV status in blood serum.

PRRSV status (positive/negative) based on blood serum samples in a particular age group from a particular herd was held up against the prevalence of clinically ill pigs in that herd's age group (see Figure 21). If the prevalence of clinically ill pigs (defined through "respiratory distress" in the form of coughing) was above 3%, then that herd's age group would be defined as clinically sick. Data from all three herds and age groups was combined, and Fisher's exact test was used to test for difference between signs of clinical illness and PRRSV status in blood serum.

Results			
	Clinical sick +	Clinical sick -	*Marginal Row Totals*
PRRS +	2	4	6
PRRS -	1	2	3
Marginal Column Totals	3	6	9 (Grand Total)

Figure 21. Fisher's exact test for comparison of clinical symptoms and PRRSV status.

The result from Fisher's exact test is: p = 1.00 (no statistically significant difference).

Discussion

In this master's thesis, we examined the dynamics, persistence/occurrence and genetic characteristics of PRRSV in three Danish swine herds presumed to be stable (PRRSV antibody positive yet weaning PRRSV negative piglets). Interesting findings across all three herds were:

1. None of the three herds followed McRebel completely.
2. A low occurrence of clinical symptoms related to PRRSV was found in all three herds, and no relation between clinical illness/symptoms and PRRSV positive blood serum was found.
3. No samples from 0 weeks old pigs (PUCS and tonsil swabs) or air samples in general were found to be PRRSV positive.
4. One herd was found to have PRRSV positive blood serum in 4 weeks old pigs, two herds were found to have PRRSV positive blood serum in 8 weeks old pigs.
5. All three herds had PRRSV positive blood serum and tonsil swabs based on the pooled samples consisting of single samples collected from the same 12 weeks old pigs. Ct-values from blood serum were significantly lower than Ct-values from tonsil swabs.
6. All samples sent for Sanger sequencing (ORF5) showed considerable similarity with Ingelvac PRRS® MLV (herd 1 samples 99.50%, herd 2 samples 98.84-99.17%, herd 3 samples 98.84-99.00%). Furthermore, no quasispecies were found using NGS Ion Torrent.

The following is a discussion of these findings.

1 Discussion of findings

The results from age group 0 weeks (not PRRSV positive) either indicate that truly none of the piglets at 0 weeks of age was PRRSV positive or that our sampling methods or sample size are insuffi-

cient. If we assume that the samples were truly negative, it tells us that the sows produce non-viremic piglets and that the herds have a stable sow team. To our knowledge, PUCS samples have not previously been used as a diagnostic method to detect PRRSV, but studies on PCV2 have shown that PUCS samples are a more sensitive method than colostrum (Seate, J. et al., 2016). Regarding how PUCS samples were taken, the placentas were between 1 and 2 days old when collected, which could have decreased the amount of virus in the umbilical blood to an undetectable level. Concerning sample size, it is possible that the prevalence of PRRSV is so low that our sample size is not sufficient to detect PRRSV through PUCS (sample size at 42 samples). Looking at tonsil swabs, the way these was taken could also be a problem (e.g. not swabbed the tonsils properly). Furthermore, another problem could be that newborn piglets do not contain PRRSV in their tonsils or that the amount of PRRSV in the tonsils is not high enough to be detected. The assay used for detecting PRRSV in the tonsil swabs was the Kleiboeker Mod-1 (Kleiboeker, S. B. et al., 2005; Wernike, K. et al., 2012). The sensitivity of the assays for both PRRSV type 1 and type 2 was found to be less than a single $TCID_{50}$ (correlates to 5–10 RNA molecules), and it is not significantly reduced when the real time RT-PCR is performed in a multiplex format (Kleiboeker, S. B. et al., 2005). It has not been possible to find any studies, which have validated the use of tonsil swabs in newborn piglets for PRRSV detection. Studies have shown that PRRSV can be isolated from blood serum of newborn piglets born by PRRSV positive sows (Benfield, D. et al., 2000; Christianson, W. T. et al., 1993). This suggests that vertical transmission is highly possible, however, it is unknown if the sensitivity in PUCS is as high as it is in blood serum. Nonetheless, collecting blood samples from newborn piglets is quite invasive and possibly dangerous, thus, further studies on PUCS samples as a diagnostic method for detecting PRRSV could be of great value.

All air samples were negative for PRRSV. This air sampling method has previously been tested on poultry (campylobacter) and cattle (foot and mouth disease) (Christensen, L. S. et al., 2011; Olsen, K. N. et al., 2009). Several studies have indicated that PRRSV can be transmitted through air (Kristensen, C. S. et al., 2004; Dee, S. et al., 2009; Otake, S. et al., 2010). It has been demonstrated that airborne transmission of PRRSV could happen between pig units when only 1% of the air from a PRRSV positive unit reached (through a ventilation system) a PRRSV negative unit (Kristensen, C. S. et al., 2004). When comparing the negative air samples to our blood serum and tonsil swab samples, we found that they did not correspond since some of the blood serum and

tonsil swab samples were positive. This suggests that the air sampling method is not sensitive enough to detect PRRSV in the air. However, we did observe that the Ct-values in blood serum samples overall were quite high suggesting that the pigs were not highly viremic and thereby the amount of virus in the air possibly was not high enough to detect PRRSV. Furthermore, the air samples were not purified before tested with real time RT-PCR, and it is not validated without extraction/purification. Thus, validation is needed before AeroCollect can be used as a reliable detection method for PRRSV.

1.1 Findings in herd 1

Herd 1 tested positive in five out of six pools (blood serum and tonsil swabs) in the age group 12 weeks of age suggesting that PRRSV is circulating in the nursery. Based on the answers from the questionnaire, nothing suggested an obvious breach of the McRebel strategy. However, regarding drying the section after washing, they only air-dry it for minimum 24 hours. Studies have shown better survival of PRRSV in cold (140 hours at 4 °C and 20 hours at 21 °C) and moist environment, which could maintain the virus in this herd (Bloemraad, M. et al., 1994). Another possible risk factor is air transmission of PRRSV from one section to another maintaining an ongoing infection (Kristensen, C. S. et al., 2004). Furthermore, hygiene measures are not met as the staff never washes hands or boots (Otake, S. et al., 2001). Moreover, during our visits to the herd, we observed that there was no strict routine regarding movement between the sections as we were allowed to move from the oldest pigs to the youngest pigs without any restrictions. Thus, there is a potential risk of the staff carrying PRRSV between the sections and thereby maintaining an ongoing infection.

1.2 Findings in herd 2

Herd 2 tested PRRSV positive in four out of six pools (blood serum) in age group 8 weeks, and six out of six pools (blood serum and tonsil swabs) in age group 12 weeks. This suggests that the infection only circulates in the nursery. Based on the answers from the questionnaire, the spread of PRRSV could be due to the fact that they do not always follow AI/AO principles, which means that younger and susceptible pigs are introduced to an environment with older and possible infected pigs. The reason for this strategy is that the herd produce an overload of pigs. They have recently started to sell 7 kg and 15 kg pigs to create more space in order to follow AI/AO. Looking at the

positive aspects, this is the only herd that washes boots and hands between each unit and thereby attempts to prevent PRRSV spread from one unit to another (Otake, S. et al., 2001). Like in herd 1, a possible risk factor is air transmission of PRRSV from one section to another maintaining an ongoing infection (Kristensen, C. S. et al., 2004). Most likely, PRRSV persisted in this herd because of the breach of McRebel by not following the AI/AO principles.

1.3 Findings in herd 3

Herd 3 tested PRRSV positive in three out of six pools (blood serum) in age group 4 weeks, two out of six pools (blood serum) in age group 8 weeks, and six out of six pools (blood serum and tonsil swabs) in age group 12 weeks. This was the only herd that tested PRRSV positive in 4 weeks old pigs. It is not clear whether the piglets are infected in the farrowing unit or shortly after they are moved to the nursery. The staff weans on Saturdays, and the blood samples were collected on a Wednesday. Thereby, the pigs had been in the nursery for 5 days. Experiment has shown that viremia can be found from day 3 PI (Yoon, I., 1993), and in light of this, the pigs might already become infected in the farrowing unit, but it is also possible that the pigs were infected in the nursery section. If the pigs were infected in the farrowing unit, then PRRSV can originate from multiple sites. One scenario is that the sows were PRRSV positive and thereby not stable. We did not find any positive test results (PUCS or tonsil swabs) in the youngest pigs from the farrowing unit, and furthermore, no productivity data strongly indicated that sows were PRRSV positive. However, it is possible that the prevalence of PRRSV in the young piglets was very low, and therefore, the number of samples taken was not sufficient to detect PRRSV (sample size = 60 samples for tonsil swabs and 16 samples for PUCS). Kristensen et al. had a similar herd description like herd 3 with an ongoing PRRSV infection in the nursery unit (Kristensen, C. S. et al., 2014c). They assumed that the sow team was stable, but to be sure whether the PRRSV came from the nursery unit or the farrowing unit, nose swabs were taken from all piglets within two weaning teams with 4 months apart. The results showed that even though all of the first samples were negative, piglets in the second weaning team were PRRSV positive indicating that the sow team was not stable as assumed. In context to our study, one might suspect that even though we had a large sample size, if we took new samples, we might get positive results. To be sure that the sow team is stable, blood samples could have been collected from the sows and tested using real time RT-PCR. Another scenario is that the sows are

indeed stable. This implies that PRRSV is circulating between the piglets in the farrowing unit and that they became infected at some point before weaning. Herd 3 has sectioned the farrowing unit, and according to the questionnaire, they follow AI/AO principles. A possible factor influencing the introduction and constant circulation of PRRSV in the farrowing unit is that none of the staff members washes hands and/or boots or changes boots between each unit. They have the same outfit on in the nursery as they have in the farrowing unit. The staff can therefore carry PRRSV from infected to susceptible pigs (Otake, S. et al., 2001). They claimed that they followed AI/AO in the farrowing unit, but as described above, we cannot be completely sure that all staff members always follow it. If they move older pigs, which are too small to be weaned, into litters of younger piglets, they risk infecting susceptible pigs. Several McRebel guidelines were not followed inside the farrowing unit, which is a problem, when PRRSV is not under control. Cross-fostering starts earlier than 24 hours after farrowing (as recommended) but continues for up to 3 days. Cross-fostering can pose a threat of spreading the virus to other litters. Newly weaned piglets (from sows used as a nurse sow) are not moved immediately to the nursery. Piglets with viremia early in life can shed virus at any time, even while they stay with the sow. Murtaugh et al. showed that the load of virus is kept reasonably under control as long as the piglets remain with the sow and are protected by maternal antibodies (Murtaugh, M. P. et al., 2010). Nevertheless, there is a higher number of PRRSV positive pigs at weaning than at farrowing. This means that it is a combination of age and decreasing maternal protection that make PRRSV positive piglets more infectious at weaning. At the same time, PRRSV negative pen mates will also lose maternal protection (due to age) and become more susceptible for PRRSV (Murtaugh, M. P. et al., 2010; Morrison, R. B. et al., 1996). Furthermore, stress associated with weaning worsens the infection, and all this leads to the argument of not having weaned pigs in a farrowing unit.

The positive results from 8 and 12 weeks of age pigs suggest that the PRRSV circulates in the entire nursery. A reason for this could be that they do not follow the McRebel guideline regarding AI/AO principles and thereby risk introducing virus from older pigs to younger susceptible pigs. Furthermore, spread of PRRSV through air transmission from one section to another and thereby maintaining an ongoing infection poses another risk (Kristensen, C. S. et al., 2004). Another explanation of the positive results in 8 and 12 weeks of age pigs could be that the pigs are already infected at 4 weeks of age and then carry the infection with them through the nursery.

1.4 Clinical signs of PRRSV

Based on the questionnaire, the clinical signs differed among the 3 herds as PRRSV is known to do (Christianson, W. T. et al., 1993; Collins, J. E. et al., 1992). In two of the herds, the staff observed mild clinical signs of PRRSV, while the third herd never observed any clinical signs. Herd 1 had a high number of piglets/year sow, which can explain the low weight at weaning (2.1 kg lower than national average). Many live borns can result in a low birth weight and thereby leads to low weight at weaning (Quiniou, N. et al., 2002). Also, herd 2 had low weight at weaning (1.38 kg lower than national average) and a slightly higher dead until weaning (1.16% higher than national average). Whether the mortality was due to PRRSV, other infections or management related issues is unknown. Herd 3 had slightly elevated number of stillborn/litter, markedly high dead until weaning (6.5% higher than national average) and a poor daily gain (36 g less than national average). These are all typical clinical signs of PRRSV.

Based on clinical registrations in the age groups 4, 8 and 12 weeks, a prevalence of symptoms was calculated (unthrifty, respiratory distress and blobs of diarrhea) in each age group and in each herd. Prevalences (<5%) of unthrifty and respiratory distressed pigs as well as the occurrence of diarrheal blobs were low, although one group of 4 weeks old pigs in herd 3 had a high prevalence of sneezing (90-100%). However, this high prevalence was not observed later in the nursery, and therefore, it was not considered to have any importance in relation to PRRSV.

To determine if there is any association/relation between PRRSV found in blood serum and prevalence of clinically sick pigs (in our study based on a prevalence of respiratory distress symptoms above 3%), Fisher's exact test was used. We did not differentiate between age groups or herds in order to have as much data as possible. The calculated prevalences from the clinical registrations were low, so there was no expectation that the Fisher's exact test would show any difference between these two covariates. This is in accordance with the fact that vaccination decreases clinical symptoms of PRRVS in pigs (Linhares, D. C. L. et al., 2011). Indeed, all of the herds had been vaccinated with Ingelvac PRRS® MLV, and furthermore, we found through Sanger sequencing that all three herds had the vaccine strain circulating.

1.5 Sanger sequencing

Sanger sequencing of the ORF5 showed that in all three herds the virus type circulating was highly similar with Ingelvac PRRS® MLV (ranging from 98.8-99.5%). This makes sense since that vaccine was used in all three herds. This is in accordance with previous studies, where it was found that the Danish PRRSV type 2 ORF5 has little diversity (Kvisgaard, L. K. et al., 2013a). The results from the Sanger sequencing showed very little intra-herd variation (samples clustering together). However, a small inter-herd variation was seen. This is interesting in a diagnostic perspective, since it appears that ORF5 is slightly different between the swine herds.

1.6 Next-Generation Sequencing

Because only parts of the genome were sequenced, it was not possible to look for quasispecies in the whole genome. The reasons for not obtaining full genome PCR fragments could be low concentration in the isolate, bad quality of extracted RNA or variation in primer binding site. Because PRRSV is highly heterogeneous, it can be a challenge to find conserved regions, where primers can bind. In addition, the sample size and the distribution of the samples should have been larger and more equality divided between age groups, respectively. However, this was not possible in our study due to the lack of appropriate sample material (very few positive samples in age groups 4 and 8 weeks of age).

Surprisingly, our NGS Ion Torrent sequencing showed very little variation in the fragments and only in the form of single nucleotide deletions or a few single nucleotide variations. Thereby, we found no quasispecies. The sequencing was performed on PCR products, and mutations located in the primer region might not be discovered. Yet techniques where sequencing is performed directly on viral RNA have been developed (Lu, Z. H. et al., 2014; Lu, Z. H. et al., 2014a). Development and evolution of quasispecies in a PRRSV strain depend on whether the mutation makes it more compatible in the environment (Lauring, A. S. et al., 2010). In our study, we found that all three herds had strains very similar to the Ingelvac PRRS MLV strain probably brought to the herds through blitz vaccination. The latest vaccination of the herds was performed within the last year, and it is thereby reasonable that the sequences obtained showed very little variation. Indeed, studies using PRRSV modified live vaccines grown in cell cultures have shown that it is possible to create a population of minor variants (Zhao, P. et al., 2012).

When comparing the NGS consensus sequences (both reference and de novo) with the corresponding ORF5 Sanger sequences, they were found to be 100% identical. Thus, it indicates that the Sanger sequencing was just as accurate as the NGS Ion Torrent regarding ORF5. However, NGS is still the preferred method since it gives a more comprehensive sequencing (massive parallel sequences of much longer fragments).

2 Future prospects and recommendations

Our recommendations to the herds in order to obtain control over or eliminating PRRSV are elaborated without any further considerations of the herds' economical and practical possibilities.

2.1 Herd 1

As the problem in this herd only lies in the nursery (age group 12 weeks), it should be possible to eliminate the PRRSV from the herd. One strategy could be to follow AI/AO principles strictly and thereby "push" the virus out of the herd. All McRebel guidelines should be followed completely such as movement within the herd (youngest → oldest pigs) and hygiene measures (washing of hands/boots). Thereby the circulation of virus should be stopped. Another strategy could be to depopulate the nursery, clean and disinfect all the sections and repopulate with PRRSV negative pigs.

It is possible to be declared a PRRSV negative herd in two different ways. One is through spontaneous sanitization (virus is pushed out only by optimizing management strategies), where 30 blood samples from 12 weeks old pigs are tested negative for PRRSV antibodies with ELISA, and the herd gets a "sanitization status". The herd is declared PRRSV negative if 30 new blood samples are also tested negative for PRRSV antibodies (SPF, 2009). The other way is through depopulation, and one month after that, a minimum of 30 blood samples from 12 weeks old pigs are tested with ELISA to check for virus antibodies. In addition, a second set of minimum 30 blood samples are ELISA tested again six months after the first set. If all these blood samples are PRRSV negative, the herd is declared PRRSV negative (SPF, 2009).

2.2 Herd 2

In this herd, PRRSV circulates in most of the nursery (8 and 12 weeks old pigs), and it should therefore be possible for this herd to eliminate PRRSV as well. One of the problems in this herd is that

they have an overload of pigs in relation to the facilities, and AI/AO principles can therefore not be followed completely. Many of the recommendations to herd 1 also applies for this herd. However, the staff at herd 2 already washes hands and boots between units, and one optimizing approach could therefore be to expand this hygiene strategy to wash hands/boots between sections. Shortly, as for herd 1, herd 2 could also try to either follow McRebel strictly ("spontaneous sanitization") or depopulate the nursery (SPF, 2009).

2.3 Herd 3

In this herd, PRRSV circulates in the entire nursery and maybe in the farrowing unit as well (4, 8, 12 weeks old pigs). Firstly, this herd has to figure out whether the infection of the 4 weeks old pigs originates from the sows (unstable sow team), as contamination within the farrowing unit or after weaning. This can be done by taking blood samples from the sows and testing them with real time RT-PCR to detect virus. If the sows are PRRSV positive, the strategy could be to blitz vaccinate the sows twice with Ingelvac PPRS® MLV with 4 weeks between each vaccination. Experiments have shown different results regarding the duration of viremia after vaccination (see Table 13) ranging from 21 dpv (days post vaccination) to 62 dpv (Martinez-Lobo, F. J. et al., 2013; Mengeling, W. I. et al., 2003; Nielsen, T. L. et al., 1997; Kristensen, C. S. et al., 2016).

Reference	Viremia (days post vaccination)
Martinez-Lobo, F. J. et al., 2013	**21**
Mengeling, W. I. et al., 2003	**42**
Nielsen, T. L. et al., 1997	**21**
Kristensen, C. S. et al., 2016	**62**

Table 13. Studies on duration of viremia after vaccination with Ingelvac PRRS® MLV.

This means that a few sows can be viremic up to 9 weeks after vaccination. Therefore, it could be necessary to wait for a period of 13 weeks before blood samples are collected from weaners and tested for PRRSV to be sure that no transmission of PRRSV happened from the viremic sows to the piglets (9 weeks until the sows are non-viremic and 4 weeks until piglets become weaners). However, if the scenario is that the sows are PRRSV negative (the sow team is stable), and the piglets are infected within the farrowing unit, the focus should be on following the McRebel guidelines in rela-

tion to cross-fostering (earlier than 24 hours after farrowing) and move the newly weaned piglets directly to the nursery. Furthermore, optimal hygiene strategies should be followed so that the infection is not carried from the nursery to the farrowing unit. These actions should be able to "push" PRRSV out of the farrowing unit. If the infection originated in the nursery, it should be possible to eliminate the virus from the herd by following McRebel guidelines strictly (e.g. following AI/AO principles) and following more comprehensive hygiene strategies (wash boots and hands in general) (SPF, 2009).

3 Discussion of study design and methodology

3.1 Diagnostic tools

In our study, we chose to pool samples. The advantage of pooling is that many samples can be tested fast at a lower cost. The pools were screened using a real time RT-PCR, which is a highly sensitive method but the reliability is questionable when used on pooled samples. Pooled samples may be diluted (depending on the number of single samples pooled and the virus load in the single samples) which may decrease sensitivity of real time RT-PCR (Muniesa, A. et al., 2014). Pooling single samples can possibly dilute the virus load to a level under the detection limit, thus creating false negatives. It has previously been shown that up to 14% of positive samples can be missed if the samples are collected within the first 5 days of infection and pooled in five at the same time (Gerber, P. F. et al., 2012; Rovira, A. T. et al., 2007). In context to our study, samples from 4 weeks old pigs were pooled in 10. Three pools from herd 3 were found PRRSV positive, however, we chose only to test individual samples from one pool. The results from this showed that only two pigs were positive, which means that they must have had a high virus load in order for the pooled sample to be PPRSV positive (Ct-value <40).

Tonsil swabs were chosen as a part of the samples strategy because it is known from the literature that PRRSV persists in lymphoid organs with a high viral load in the initial phase of infection and can be isolated from oropharyngeal scraping up to 157 days PI (Duan, X. et al., 1997; Wills, R. W. et al., 1997a). Tests of blood serum and tonsil swabs from the 12 weeks old pigs showed that they are equally good at detecting virus. However, when comparing Ct-values (regardless of herds) between pooled blood serum and pooled tonsil swabs, we found that pooled blood serum samples had a significantly lower Ct-value ($p = 0.003$) than the pooled tonsil swab samples.

This indicates that blood serum seems to be a more sensitive sampling method than tonsil swabs for PRRSV detection. Previous experiments have shown positive results in both blood serum and tonsil swabs from the same PRRSV infected pigs (Madapong, A. et al., 2016; Rosengren, L. et al., 2011). This means that tonsil swabs are an acceptable method to detect PRRSV. Nonetheless, in accordance with our findings, one study has previously shown that blood serum has the highest sensitivity for detecting PRRSV (Gerber, P. F. et al., 2012). In context, we experienced that collection of blood serum and tonsil swabs are equally stressful for the pigs, and even though tonsil swabs are non-invasive, we believe that blood serum samples should be preferred as a method to detect PRRSV due to their higher sensitivity.

3.2 Next-Generation Sequencing

For the NGS, a Trizol purification protocol was used. This was done to obtain a better quality of RNA for NGS. We found that only one sample (B1-s100(E3)) had good RNA quality (260/280 ratio of 2.94). The other samples had a low ratio, which could indicate the presence of protein, phenol or other contaminants that are absorbed strongly at or near 280 nm. Furthermore, small pH changes in the 260/280 making an acidic environment can lower the ratio by 0.2-0.3. The ratio of different nucleotides (G, A, U, T, C) can also affect the 260/280 ratio (260/280 and 260/230 ratios, 2017). The lower quality of RNA obtained through Trizol purification could have negatively influenced our NGS results.

Furthermore, the two different propagation methods were tested on the same Trizol eluates. The results showed that it was not possible to obtain good quality sequences using method 2 (see Methods and materials). The reason for this could be that the concentration of PRRSV in the samples was too low. Furthermore, the primers added in the reaction were not specific and could thereby bind to any DNA in the samples. This could cause contaminated products to be amplified which was the case in one of the samples where DNA from homo sapiens was obtained. Thereby, method 2 was not good at obtaining full genome PRRSV sequences.

3.3 Study design

The study only included three herds, and it is therefore difficult to comment on the general situation of PRRSV occurrence, persistence and dynamics in all Danish herds.

The study was designed as a cross-sectional study which provided a snapshot of the PRRSV situation in the three case herds. This design allowed us to compare many different variables at the same time, e.g. PRRSV presence in different age groups. However, in order to give a more clear picture on PRRSV dynamics, a longitudinal study would have been preferable since this would allow us to follow the exact same pigs over time and determine onset of PRRSV infection. Yet, if this kind of study design had been chosen, it would have taken about 12 weeks only to collect all the blood samples from the youngest piglets (just after farrowing) to the oldest pigs (12 weeks old), and this could not be done with our time schedule. Therefore, a cross-sectional study was chosen.

To provide a more uniform view of the PRRSV situation in the case herds, the inclusion criteria could have been even more specific. For example, all herds could have had the same status (SPF/conventional) and herd size (number of sows ranging from 1,000-1,200). This would have allowed us to make a better comparison of various parameters between herds.

Furthermore, a more comprehensive understanding and a more accurate prevalence of PRRSV could have been reached if all the collected samples were tested individually (as single samples). However, the budget and time schedule did not allow this.

3.4 Sample size and collection strategy

Results from another study have shown that in herds with low clinical signs of PRRSV, the preferred age group to detect PRRSV in is age groups 9 and 16 weeks since these had the highest odds ratio for PRRSV detection (Duinhof, T. F. et al., 2011). Duinhof et al. calculated the sample size (with 95% confidence intervals) based on an estimated virus prevalence (of 21-40%) for detecting PRRSV to be 13. In this master’s thesis, a prevalence of 10% in the oldest pigs (8 weeks and 12 weeks old) was used to be sure that as many positive pigs were found as possible and therefore the sample size in these age groups was 30. The PRRSV prevalence at the youngest pigs (0 weeks and 4 weeks old) was estimated to be 5%, which increased the sample size to 60. These prevalences are estimated and since we tested pooled samples (and not individual samples), we do not know what the actual prevalence of PRRSV is in our three case herds. One could argue that the estimated prevalences used for the calculation of sample size could be set to 20% (oldest pigs) and 10% (youngest pigs) (Duinhof, T. F. et al., 2011). This would reduce the number of samples with 50%, and the budget might then have allowed us to test all samples individually. The aim of this study was not to

show the true prevalence of PRRSV in a Danish swine herd but just to investigate whether or not PRRSV was present.

The strategy for sample collection was to collect from several litters/sections with few samples from each litter/section. The transmission of PRRSV from pig to pig is not always very efficient. A study has shown that direct contact between pigs only infected 2 out of 8 pen mates with PRRSV, and furthermore, none was infected by aerosol exposure (Torremorell, M. et al., 1997). Rose et al. showed that vaccinated piglets have viremia for a shortened period (Rose, N. et al., 2015). In addition, compared to a group of non-vaccinated piglets (all PRRSV positive when tested), only one from the group of vaccinated piglets was found PPRSV positive. Furthermore, this study estimated that the PRRSV transmission rate was 10 times lower in vaccinated piglets than in non-vaccinated piglets. These findings suggest that even though some pigs are PRRSV positive and potentially can transmit the virus to other pigs, it is not certain that it will happen, and this risk would be even lower if the pigs are vaccinated. The piglets (0 weeks old) in our study were not vaccinated but only had antibodies against PRRSV from the sow (maternal antibodies), but we assume that these two ways of virus protection (vaccination versus maternal antibodies) are identical. Therefore, when taking samples from many litters/sections with few samples within each litter/section, we attempted to eliminate the bias of not finding those pigs being PRRSV positive. Furthermore, the different age groups in the nursery from all three herds were spread out in several sections, so our sample strategy would also eliminate the bias of PRRSV circulating in only one of the sections but not in the other sections.

3.5 Validity of the results

Results based on answers from the questionnaire could be biased since the answers are subjective (based solely on the staff's own opinions and beliefs) and have not been confirmed through control visits. However, the productivity data was based on registrations and thus more objective and reliable.

Errors could have happened during sample collection, handling and laboratory procedures (e.g. hygiene, handling and transportation of the samples). During the laboratory process, samples were continuously frozen and thawed and this could have influenced the Ct-values negatively by raising the Ct-values and thereby make PRRSV undetectable in real time RT-PCR.

Conclusions

Based on this master's thesis, we found that in presumed stabilized swine herds, PRRSV was present (at ages 12 weeks in herd 1; 8 and 12 weeks in herd 2; 4, 8 and 12 weeks in herd 3). We found that none of the case herds followed McRebel completely, and only herd 3 had signs of PRRSV based on their productivity data (higher stillborn per farrowing, lower weight at weaning, higher death until weaning) compared with the national average. Furthermore, regardless of age group, low prevalence/occurrence of clinical symptoms were seen in all three herds suggesting that the number of clinically sick pigs cannot be used as an indicator for the presence of PRRSV. No PUCS samples or tonsil swabs (collected at age 0 weeks) were found positive suggesting that the sow teams were stable. No air samples were found positive suggesting that this detection method is not accurate enough. Moreover, in 12 weeks old pigs, we found that pooled blood serum samples had significantly lower Ct-values than pooled tonsil swab samples suggesting that blood serum might be better for PRRSV detection. Sanger sequencing of PRRSV type 2's ORF5 showed that the samples from all three herds had a high similarity to the Ingelvac PRRS® MLV with a low intra-herd variation. However, a small inter-herd variation was seen. Looking at fragments obtained from NGS Ion Torrent, no quasispecies were found, and the samples' ORF5 segment was 100% identical with the ORF5 sequences found through Sanger sequencing.

Perspectives

Further studies of PRRSV are necessary to get a more comprehensive picture of PRRSV's dynamics, persistence and genetic characteristics in Danish swine herds. A longitudinal study could have been preferred. In that way, we could have seen when the growing pigs become PRRSV positive. Including more PRRSV type 2 positive herds in such a study, we could also have investigated how many of these herds has the modified live vaccine strain circulating.

In addition, we believe that it would be beneficial to validate the use of PUCS samples and tonsil swabs in 0 weeks old pigs for PRRSV detection. This could be done by infecting the sows, collecting their placentas (and quantify the amount of virus) and swabbing the piglets' tonsils (e.g. within 1 day of age). The infection of the piglets could then be confirmed by blood serum samples. Furthermore, in our opinion, it would be beneficial to put a large effort in validating the use of

AeroCollect (air samples) since this diagnostic method would be fast, easy and economically advantageous.

To make a more comprehensive study of different quasispecies between age groups, a larger sample size should be used and the study could be carried out in both herds positive for the wild type 2 and herds positive for the vaccine type. Furthermore, a longitudinal study where PRRSV type 2 infected pigs (wild/vaccine type) were followed over time could be carried out in order to investigate the development of quasispecies.

We saw that none of the case herds had succeeded in pushing PRRSV out of the herd. Future prospects could therefore be to investigate other strategies (such as depopulation) to see if they would be better at eliminating or getting control over PRRSV. Furthermore, studies on McRebel guidelines could be done in order to find out which guidelines are more important. Hereby, it would be possible to prioritize the individual guidelines.

In our study, we have looked at eradication and vaccination strategies, internal biosecurity issues and made a genetic characterization of the virus present in the three swine herds. Another perspective could be to focus on host genetics promoting natural resistance through gene variation.

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Appendix

Appendix 1 – Questionnaire

Herd 1

Generally

Herd size (numbers of sow and gilts): 1560

SPF status: Blå + Myc + PRRS

When was PRRS discovered: 2012

When did the herd started to sanitized for PRRS: Januar 2013

When took the first vaccination place for PRRS: 2012

Which dose was used (vaccination): 2 ml

Which vaccine was used: Ingelvac PRRS

How many times were the herd vaccinated: First time in 2012 and after 2 time pr. year.

Which animals were vaccinated: Sows and gilts

Do you see other diseases in the herd: No

Is the farrowing unit built in a way so the herd can have sectioned farrowing teams (is there any half-wall between the farrowing teams)?: No

Describe the plan that was made to be free of PRRS: Vac. all sows and pigs in klima, make klima empty for 4 weeks (wash, disinfect).

1. PRRS problem:

1.1. Has there been any clinical sign of PRRS in the herd?

_____ Yes

__x__ No

1.1.1. Which clinical signs have been observed? (describe)

____

1.2. Are the PRRS problems occurred in connection with a specific event (herd enhancements / changes in management or otherwise)? (Describe)

No

1.3. How do you experienced PRRS right now in the herd?

__X__ No signs of PRRS in the herd besides positives blood samples

_____ Rare cases of piglets/weaned pigs which shows symptoms (unthrifty, respiratory distress)

_____ Always some few piglets/ weaned pigs that have clinical signs of PRRS

2. Environment

2.1. Is there floor heating in the piglets corner?

__X _ Yes

_____ No

2.2.When do the floor heating/heating lamps going to be switched on and off?

On: 2 days before farrowing

Off: 10 days after farrowing if pigs are big

3. McRebel

3.1. When do you start with cross-fostering after farrowing?

_____ Before 24 hours

__X__ After 24 hours

3.1.1. How long time after farrowing do you cross-foster? (describe)

____

3.2. Do you have nursery sows?

__X_ Yes

_____ No

3.2.1. What happens with the piglets that are weaned when making nursing sow?

____ They stay in the same farrowing section until weaning day

__X__They go to nursery section

3.3. Is the nursery section completely empty when "new" pigs come in (all in/all out)?

__X__ Yes

_____ No

3.3.1. You never move newly weaned pigs into a nursery section where older pigs are going?

_____ Yes

__X__ No

3.4. What do you do with small weak piglets at weaning?

_____ Euthanized (kill) if it assessed that the piglet will not get better

_____ the piglet will be moved to a "opsamlingsso" in the same section

__X__ the piglet will be moved to a "opsamlingsso" in the different section

_____ they are going to be weaned anyway

4. Hygiene

4.1. How do you clean the farrowing section between each farrowing team?

_____ no wash

__X__ wash without soap

_____ wash with soap

4.2.How do you dry the farrowing section after washing

__X__ by air (+ ventil)

__X__ heat canon

_____ other (describe)

____

4.2.1. How long does the section dry out? (describe)

Min. 12 hours

4.3.Do you use disinfection after washing the farrowing section?

__X__ Yes

_____ No

4.4. How do you clean the nursery section between each weaning team?

_____ no wash

__X__ wash without soap

_____ wash with soap

4.5. How do you dry the nursery section after washing

__X__ by air (+ ventil)

__X__ heat canon

_____ other (describe)

____

4.5.1. How long does the section dry out? (describe)

Min 24 hours

4.6. Do you use disinfection after washing the nursery section?

__X__ Yes

_____ No

5. Hygiene of aids

5.1. How often do you wash boots/hands in the stable?

____ between each section in the same stable (farrowing, nursery etc.)

____ between each stable (farrowing, nursery etc.)

____ when it is remembered

__X_ Never

_____ other (describe)

5.2.How often do you change needle (treatments)?

_____ between each pen

__X__ between each section

_____ when the needle is blunt

5.3.How often do you clean the instrument for tail docking?

__X__ between each piglet

_____ between each litter

_____ after all the piglets have been tail docking

_____ never

5.4. How do you clean the instrument for castration? (describe)

Wash with water and sprit

5.4.1. How do you clean the instrument for castration?

__X__ between each piglet

_____ between each litter

_____ after all the piglets have been castrated

_____ never

Herd 2

Generelt:

Besætningens størrelse (antal årssøer inkl. gylte): 700 stk.

Besætningens produktionsform: 30 kg produktion (dog med salg af 3500 7 kg grise og 5800 10-16 kg grise pga. pladsmangel i klimastalde)

SPF-status: SPF + Vac

Hvornår blev PRRS opdaget: Den blev bevidst vaccineret ind i den nye besætning som vi indkøbte i 2012 (polte, søer, gylte). Dog startede problemer hos smågrise at opstå i starten af 2014.

Hvornår startede besætningen med at blive fri for PRRS: Fra foråret 2016 har vi forsøgt at skubbe PRRS ud, dog ikke lykkedes endnu.

Hvornår blev der vaccineret for PRRS: Indkøbte polte bliver og er blevet vaccineret ved ankomst til karantænestald, og revaccineret 3 uger efter. Vi har af flere omgange vaccineret pattegrise i farestalden også, det stoppede vi dog med i foråret da farestalden blev sektioneret. Søerne har fået et boost med vaccine i foråret 2 gange med 4 ugers mellemrum.

Hvilken dosis blev der vaccineret med: Polte/gylte/søer 2 ml, Pattegrise 1 ml

Hvilken vaccine blev der anvendt: Ingelvac PRRS

Hvilke dyr blev vaccineret: Polte, pattegrise, søer/gylte

Hvor mange gange blev der vaccineret: Polte (2 gange), Smågrise (1 gang), Søer/gylte (2 gange)

Ses der andre sygdomme i besætningen: I juli 2014 konstaterer laboratoriet i Kjellerup Cytomegalovirus i vores besætning, om den har betydning for nogle af vores problemer vides ikke

Er farestalden opbygget på en sådan måde, at der kan køres sektioneret holddrift (eller findes der halvmure mellem hver holdsektion)?: Ja vi har fået sektioneret vores farestald i foråret/forsommer 2016.

Beskriv den plan som blev lagt for at blive fri for PRRS

Se det vedhæftede dokument 'Handlingsplan PRRS'

1. PRRS problem:

1.1. Har der været kliniske tegn på PRRS i besætningen? (Sæt kryds)

_x_ Ja

Nej

1.1.1. Hvilke kliniske tegn blev der set? (Beskriv)

Svækkede grise med lav immunitet, bliver langhårede, hæver omkring øjnene, inaktive, ligger bare og er passive ude langs væggen. Høj dødelighed i farestald (20-22%) og klimastalde (8%). Grise døde/blev aflivet af følgesygedomme så som blodforgiftning, svær ledbetændelse og hjernebetændelse, eller fordi de ikke voksede og var små og utrivelige.

1.2. Er problemerne opstået i forbindelse med en bestemt begivenhed (besætningsudvidelser/ændringer i management eller andet)? (Beskriv)

Nej

1.3. Hvordan opleves PRRS lige nu i besætningen? (Sæt kryds)

_____ Der er intet der tyder på PRRS i besætningen udover blodprøverne

_____ Sjældent tilfælde hos pattegrise/smågrise_hvor de har symptomer på PRRS (utrivelige/respirationsproblemer)

__X__ Der er altid nogle enkle grise (klimastalden) der har tegn på PRRS (utrivelige/respirationsproblemer)

2: Nærmiljø:

2.1 Er der gulvarme i grisehjørner? (Sæt kryds)

_____ Ja

__X__ nej

2.2 Hvornår tændes og slukkes gulvvarme og varmelampe? (Beskriv)

Varmelampe: 1 dag inde faring tændes lampen, slukkes for de fleste kuld efter 7-10 døgn.

3. McRebel:

3.1. Hvor lang tid efter endt faring påbegyndes kuldudjævning? (Sæt kryds)

____ Før 24 timer

__X_ Efter 24 timer

3.1.1. hvor lang tid efter faring foretages der kuldudjævning. (Beskriv)

Inden for 1 døgn

3.2. Laves der ammesøer? (Sæt kryds)

__X__ Ja

_____ Nej

3.2.1. Hvad gøres der med de pattegrise som fravænnes når der laves ammeso? (Sæt kryds)

_____ Bliver i stalden til det er fravænningsdag

__X__ Flyttes fra farestalden over i klimastalden

3.3. Er klimastalden altid helt tom når der flyttes nye dyr ind (AI/AU)? (Sæt kryds)

__X__ Ja

_____ Nej

3.3.1. Der flyttes aldrig nyfravænnet grise ind til ældre grise i klimastalden? (Sæt kryds)

__X__ Ja, på grund af pladsmangel kan vi risikere at der flyttes nyfravænnede grise ind i en sektion hvor der går grise som er en uge ældre. Det sker dog ikke hver uge.

_____ Nej

3.4. Ved fravænning, hvad gøres der ved små, svage pattegrise som ikke kan fravænnes? (Sæt kryds)

__X*__ Aflives hvis det vurderes at den ikke kan klare sig

* Hvis et helt kuld grise er for små ved fravænning, så flyttes so + grise til buffersektion i farestalden, hvor de står en uge mere inden de fravænnes.

_____ Bliver flyttet til en opsamlingsso i samme sektion

_____ Bliver flyttet til en opsamlingsso i en ny sektion

_____ De fravænnes alligevel

4.Hygiejne:

4.1 Hvordan rengøres farestalden mellem hvert ugehold? (Sæt kryds)

_____ Der vaskes ikke

_____ Vask uden sæbe

__X__ Vask med sæbe

4.2. Hvordan udtørres farestalden efter vask? (Sæt kryds)

__X_ Lufttørre

__X_ Varmekanon (kun vinterhalvåret)

_____ Andet (Beskriv)

____

4.2.1. Hvor længe udtørres der efter vask? (beskriv)

2 døgn

4.3. Gøres der brug af desinficering efter vask i farestalden? (Sæt kryds)

__X__ Ja

_____ Nej

4.4. Hvordan rengøres klimastalden mellem hvert hold? (Sæt kryds)

_____ Der vaskes ikke

_____ Vask uden sæbe

__X__ Vask med sæbe

4.5. Hvordan udtørres klimastalden efter vask? (Sæt kryds)

__X__ Lufttørring

__X__ Varmekanon (vinterhalvåret)

_____ Andet

4.5.1. Hvor længe udtørres der efter vask? (beskriv)

1-2 døgn (2 døgn ungsvinestald)

4.6. Gøres der brug af desinficering efter vask i klimastalden? (Sæt kryds)

__X__ Ja

_____ Nej

5. Hygiejne af hjælpemidler

5.1. Vask af støvler/hænder inde i stalden, hvor ofte? (Sæt kryds)

__X__ Ved hver ny sektion (kun hvis man har været i stier med diarré)

__X__ Ved hver ny afdeling

_____ Når det huskes

_____ Aldrig

5.2 Hvor tit skiftes der kanyle ved behandling af pattegrise/klimagrise? (Sæt kryds)

_____Ved hver ny sti

__X__ Ved hver ny sektion

_____ Når kanylen virker sløv

5.3. Hvor tit rengøres halebranderen ved halekupering? (Sæt kryds)

_____ Efter hver pattegris

__X__ Efter hver kuld

_____ Efter alle pattegrise er halekuperet

_____ Aldrig

5.4 Hvordan rengøres instrumentet (skalpel/tang), som benyttes ved kastration? (Beskriv)

Skalpel sættes i Clorhexidinsprit efter hver gris

5.4.1. Hvor tit rengøre instrumentet? (Sæt kryds)

__X__ Efter hver pattegris

_____ Efter hver kuld

_____ Efter alle pattegrise er halekuperet

_____ Aldrig

Herd 3

Generelt:

Besætningens størrelse (antal årssøer inkl. gylte):

Besætningens produktionsform:

SPF-status: Konventionel

Hvornår blev PRRS opdaget: Kan ikke huske det præcise årstal men ca. 2002/2003

Hvornår startede besætningen med at blive fri for PRRS: For mange år siden

Hvornår blev der vaccineret for PRRS: Søerne blev vacc i 2014/2015, og klimastalde vacc flere år siden.

Hvilken dosis blev der vaccineret med: fuld dosis til søerne og halv dosis til klima

Hvilken vaccine blev der anvendt: PRRS vacc med US PRRS. Kan ikke lige huske navnet

Hvilke dyr blev vaccineret:

Hvor mange gange blev der vaccineret: søer 2 gange og klima ca. 4-5 måneder

Ses der andre sygdomme i besætningen: Ap (ondartede lungesyge)

Er farestalden opbygget på en sådan måde, at der kan køres sektioneret holddrift (eller findes der halvmurer mellem hver holdsektion)?: Ja

Beskriv den plan som blev lagt for at blive fri for PRRS:

1. PRRS problem:

1.1. Har der været kliniske tegn på PRRS i besætningen? (Sæt kryds)

__X__ Ja

_____ Nej

1.1.1. Hvilke kliniske tegn blev der set? (Beskriv)

Kastninger ect. da besætningen blev smittet

1.2. Er problemerne opstået i forbindelse med en bestemt begivenhed (besætningsudvidelser/ændringer i management eller andet)? (Beskriv)

Nej

1.3. Hvordan opleves PRRS lige nu i besætningen? (Sæt kryds)

__X__ Der er intet der tyder på PRRS i besætningen udover blodprøverne

_____ Sjældent tilfælde hos pattegrise/smågrise hvor de har symptomer på PRRS (utrivelige/respirationsproblemer)

___X_ Der er altid nogle enkle grise (klimastalden) der har tegn på PRRS (utrivelige/respirationsproblemer)

2: Nærmiljø:

2.1 Er der gulvarme i grisehjørner? (Sæt kryds)

__X__ Ja

_____ nej

2.2 Hvornår tændes og slukkes gulvvarme og varmelampe? (Beskriv)

Altid gulvvarme. Varmelamper lige inden faring og slukkes ca. 4-6 dage efter

3. McRebel:

3.1. Hvor lang tid efter endt faring påbegyndes kuldudjævning? (Sæt kryds)

__X__ Før 24 timer

_____ Efter 24 timer

3.1.1. hvor lang tid efter faring foretages der kuldudjævning. (Beskriv)

3 dage

3.2. Laves der ammesøer? (Sæt kryds)

__X__ Ja

_____ Nej

3.2.1. Hvad gøres der med de pattegrise som fravænnes når der laves ammeso? (Sæt kryds)

__X__ Bliver i stalden til det er fravænningsdag

_____ Flyttes fra farestalden over i klimastalden

3.3. Er klimastalden altid helt tom når der flyttes nye dyr ind (AI/AU)? (Sæt kryds)

__X__ Ja

_____ Nej

3.3.1. Der flyttes aldrig nyfravænnet grise ind til ældre grise i klimastalden? (Sæt kryds)

__X__ Jo

_____ Nej

3.4. Ved fravænning, hvad gøres der ved små, svage pattegrise som ikke kan fravænnes? (Sæt kryds)

__X__ Aflives hvis det vurderes at den ikke kan klare sig

_____ Bliver flyttet til en opsamlingsso i samme sektion

_____ Bliver flyttet til en opsamlingsso i en ny sektion

_____ De fravænnes alligevel

4. Hygiejne:

4.1 Hvordan rengøres farestalden mellem hvert ugehold? (Sæt kryds)

_____ Der vaskes ikke

_____ Vask uden sæbe

__X__ Vask med sæbe

4.2. Hvordan udtørres farestalden efter vask? (Sæt kryds)

__X__Lufttørre

_____ Varmekanon

_____ Andet (Beskriv)

4.2.1. Hvor længe udtørres der efter vask? (beskriv)

1-2 dage

4.3. Gøres der brug af desinficering efter vask i farestalden? (Sæt kryds)

__X__ Ja

_____ Nej

4.4. Hvordan rengøres klimastalden mellem hvert hold? (Sæt kryds)

_____ Der vaskes ikke

_____ Vask uden sæbe

__X__ Vask med sæbe

4.5. Hvordan udtørres klimastalden efter vask? (Sæt kryds)

__X__ Lufttørring

__X__ Varmekanon

_____ Andet

4.5.1. Hvor længe udtørres der efter vask? (beskriv)

1-3 dage

4.6. Gøres der brug af desinficering efter vask i klimastalden? (Sæt kryds)

__X__ Ja

_____ Nej

5. Hygiejne af hjælpemidler

5.1. Vask af støvler/hænder inde i stalden, hvor ofte? (Sæt kryds)

____ Ved hver ny sektion

_____ Ved hver ny afdeling

_____ Når det huskes

__X__ Aldrig

5.2 Hvor tit skiftes der kanyle ved behandling af pattegrise/klimagrise? (Sæt kryds)

_____Ved hver ny sti

__X__ Ved hver ny sektion

_____ Når kanylen virker sløv

5.3. Hvor tit rengøres halebranderen ved halekupering? (Sæt kryds)

_____ Efter hver pattegris

__X__ Efter hver kuld

_____ Efter alle pattegrise er halekuperet

_____ Aldrig

5.4 Hvordan rengøres instrumentet (skalpel/tang), som benyttes ved kastration? (Beskriv)

Står i sprit

5.4.1. Hvor tit rengøre instrumentet? (Sæt kryds)

__X__ Efter hver pattegris

_____ Efter hver kuld

_____ Efter alle pattegrise er halekuperet

_____ Aldrig

Appendix 2 – PRRS strategy in herd 2

PRRS-handlingsplan forår 2016

Søer:

- Blitzvaccination af alle søer og gylte
  - 1. gang fredag d. 1/4-2016
  - 2. gang fredag d. 29/4-2016

- Vaccination af polte ved ankomst til karantæne og revaccineres 3 uger efter

Farestalden:

- Alle pattegrise vaccineres på dag 10-12 frem til og med fredag d. 29/4 (start mandag d. 11/4-2016)
- Pattegrise bliver i den sektion hvor de er født (ved behov må grisene kun flyttes fremad - op til ældre grise)
- Mellemsøer: egne grise må ikke flyttes med!
- Ingen grise uden søer i farestalden (Ammesoens grise flyttes direkte i klimastalden)
- McRebel:
  - Kuldene låses efter kasteration (3-4 levedøgn)
  - Ingen opsamlingshold
  - God kanylehygiejne: Samme kanyle pr. kuld, samme kanyler må <u>ikke</u> anvendes mellem to farestaldssektioner
  - Streng styrring af farestaldsbuffer alternativt fravænnes alt!

Klimastald/Ungsvin:

- Sektioneret drift (ved behov må grise kun flyttes fremad til ældre grise)
- Alle grise vaccineres ved fravænning i de næste 8 uger (start mandag d. 11/4-2016) indtil alle grise i klimastalden og ungsvinestaldene er vaccineret **OBS!** Dog ikke de grise som er vaccineret i farestalden
- Restgrise må ikke flyttes tilbage
- Få sektion 8 i ungsvinestalden kørt med ind i normal drift, nedlæggelse af buffer!
- Ældre grise der ikke kan sælges, flyttes hurtigst muligt til Balle for at undgå smitteudskillelse og for at give plads

Appendix 3 – Calculation of sample size

Sample size to detect disease	
Population size (N)	600
Assumed prevalence (p)	0,1
Number of detectable cases (d)	60
Probability of finding at least one (P)	0,95
Required sample size (n)	27,79
Rounded sample size (n)	28

Appendix 4 – Overview of air samples

Operator	Sample ID	Age [weeks]	Comments
		HERD 1	
Operator 1	D1	4	Section 1 measurement
Operator 1	D2	4	Section 2 measurement
Operator 1	D3	8	Section 1 measurement
Operator 1	D4	8	Section 2 measurement
Operator 1	D5	12	Section 1 measurement
Operator 1	D6	12	Section 2 measurement
Operator 1	D7	4	Screening measure
Operator 1	D8	8	Screening measure
		HERD 2	
Operator 2	D9	4	Section 1 measurement
Operator 2	D10	4	Section 2 measurement
Operator 2	D11	4	Screening measure
Operator 2	D12	8	Section 1 measurement
Operator 2	D13	8	Section 2 measurement
Operator 2	D14	8	Screening measure
Operator 2	D15	12	Section 1 measurement
Operator 2	D16	12	Section 1 measurement
Operator 2	D17	12	Section 1 - Screening measure
Operator 2	D18	12	Section 2 measurement
Operator 2	D19	12	Section 2 measurement
Operator 2	D20	12	Section 2 - Screening measure
Operator 3	D21	12	Section 2 measurement
Operator 3	D22	12	Section 2 measurement
Operator 3	D23	12	Section 2 - Screening measure
		HERD 3	
Operator 3	D24	4	Section 1 measurement
Operator 3	D25	4	Section 2 measurement
Operator 3	D26	4	Screening measure
Operator 3	D27	8	Section 1 measurement
Operator 3	D28	8	Section 2 measurement
Operator 3	D29	8	Screening measure
Operator 3	D30	12	Section 1 - 1 measurement
Operator 3	D31	12	Section 1 - 2 measurement
Operator 3	D32	12	Section 1 - Screening measure
Operator 3	D33	12	Section 2 - 1 measurement
Operator 3	D34	12	Section 2-2 measurement
Operator 3	D35	12	Section 2 - Screening measure

Appendix 5 – QIAGEN OneStep kit for real time RT-PC

The mastermix was prepared in a separate room and put into 0.1 ml transparent tubes in 72-tube loading blocks, and the template RNA was then added in a separate room. Thereafter the tubes were capped and the real time RT-PCR was run on a Rotorgene Q (QIAGEN). Data processing was made on the Rotor-Gene Q software version 2.0.2 (Rotor-gene Q, 2012).

The real time RT-PCR used was a one step kit called QIAGEN OneStep RT-PCR kit (QIAGEN, OneStep RT-PCR, 2016).

All reactives were thawed before using and during the making of the mix, the other components were kept in the freezer or on a iceing block.

The master mix consisted of the following per PCR reaction:

- RNase free water (kit): 10 µl
- 5x QIAGEN OneStep RT-PCR buffer (kit): 5 µl
- dNTP mix (10 mM solution) (kit): 1 µl
- Each primer (10 µM solution) (kit): 0.75 µl
- Each probe (10 µM solution) (kit): 0.5 µl
- QIAGEN OneStep RT-PCR Enzyme Mix (kit): 1 µl
- Master mix volume: 23 µl
- Purified volume: RNA 2 µl
- Total reaction volume: 25 µl

The real time RT-PCR reaction was processed on the Rotorgene 6000/Q with the following thermal profile:

- 30 minutes at 50℃, 15 minutes at 95℃, 45 cycles of 15 seconds at 94℃, 60 seconds at 60℃, 10 seconds of 72℃.

During each primer extension cycle, a fluorescence signal was obtained. Two different pairs of wavelengths were used according to type 1 and type 2 probes:

Type 1: EU probe: Source light 470 nm, and emitted light signal 510 nm.

Type 2: NA probe: Source light 530 nm, and emitted light signal 555 nm.

Furthermore the threshold was determined to 0.01.

Appendix 6 – PRRSV ORF5 US PCR with QIAGEN OneStep RT-PCR kit for Sanger sequencing

The PCR mix is made as shown below:

For each PCR reaction PRRSV ORF 5 US
26 µl water from kit 10 µl QIAGEN OneStep RT-PCR buffer 2 µl dNTPmix from kit 3 µl PRORF5USfw [10µM] 3 µl PRORF5USrev [10µM] 1 µl QIAGEN OneStep RT-PCR-Enzyme Mix Total volume of 45 µl

All reagents were kept on ice, vortexed and spun before use except the enzymes, which were only mixed carefully, and the mixture was placed in PCR tubes. Thereafter 5 µl RNA extract was added the to mix. The samples were kept on ice until just before they were put in the PCR machine, where the PCR block was preheated to 50℃. Then the cycle runs as shown below:

- 30 minutes at 50℃, 15 minutes at 95℃, 50 times (30 seconds at 94℃, 30 seconds at 60℃, 60 seconds at 72℃), pause at 4℃.

The samples were kept at -20℃ until later use. The PCR products were run on a 2% agarose E-gel from Invitrogen with 10 µl, 100 bp ladder reference as a size marker. Expected length on the US fragment (bp) is 818.

Appendix 7 – LGC Genomics GmbH, concentrations

PCR-product	Name	Primer	Optic density ng/µl	Added H20 for a 200 ng concentration
B1-s99	156	ORF5-US	76	7 µl H2O
B1-s100	157	ORF5-US	110	8 µl H2O
B1-s108	160	ORF5-US	112	8 µl H2O
B1-t100	167	ORF5-US	82	7 µl H2O
B1-t108	170	ORF5-US	108	8 µl H2O
B2-s69	177	ORF5-US	148	9 µl H2O
B2-s105	185	ORF5-US	91	7 µl H2O
B2-s111	186	ORF5-US	153	9 µl H2O
B2-s112	187	ORF5-US	116	8 µl H2O
B2-t111	191	ORF5-US	97	7 µl H2O
B2-t112	192	ORF5-US	156	9 µl H2O
B3-s45	202	ORF5-US	143	9 µl H2O
B3-s67	209	ORF5-US	215	9 µl H2O
B3-s91	213	ORF5-US	213	9 µl H2O
B3-s95	217	ORF5-US	178	9 µl H2O
B3-s108	220	ORF5-US	262	9 µl H2O

B3-s110	222	ORF5-US	193	9 µl H_2O
B3-t109	228	ORF5-US	184	9 µl H_2O

*B1: Herd 1. B2: Herd 2. B3: Herd 3.

Appendix 8 – RNA extraction from blood serum using Trizol LS

Reactives:

- Trizol LS (Ambion: 10296010)
- 1-Bromo-3-Chloropropane (Sigma: B9673)
- RNAse-free water
- 75% EtOH (dilute fresh: 12.5 ml RNase-free water + 37.5 ml absolute EtOH)
- RNeasy mini kit for manual purification

Procedure:

The serum was filtered through a 0.45 µm sterilefilter. A mix was made with 250 µl filtered serum, 750 µl Trizol LS (in 1:3 relation of sample and Trizol LS, e.g. 100 µl blood serum sample + 300 µl Trizol LS). The sample was vortexed thoroughly and set to rest in 5 minutes. 200 µl 1-Bromo-3-Chloropropane (or 0.266 x volume of Trizol LS added, e.g. 300 µl x 0.266 = 79.8 µl 1-Bromo-3-Chloropropane). The sample was shaken thoroughly in 15 seconds and set to rest in 3 minutes. The sample was centrifuged at 12,000 xg in 15 minutes at 4℃. Then the water phase was put into a new tube. 1 x vol 75% EtOH was added and mixed by turning the tube a few times.

The RNA was purified using the RNeasy Mini kit as follows:

1. 700 µl was placed in the RNeasy column and centrifuged @8000xg, 15 seconds, RT. The run through was dispatched.
2. Point 1 was repeated until the whole sample is put on pillar.
3. 700 µl RW1 was added and centrifuged at @8000xg, 15 seconds, RT. The run through was dispatched.
4. 500 µl RPE was added and centrifuged @8000xg, 15 seconds, RT.The run through was dispatched.
5. 500 µl RPE was added and centrifuged @8000xg, 2 minutes, RT. The run through was dispatched.
6. The pillar was centrifuged until it was dry at @8000xg, 1 minutes, RT. The run through was dispatched.
7. The pillar was placed in a new Eppendorf-tube (mark sample + E1).
8. 30 µl RNase free water was added to the pillar membrane (without touching).

9. The RNA was eluted by centrifuging @8000xg, 1 minute, RT.

10. The pillar was transferred to a new tube (mrk sample + E2), and points 8 and 9 were repeated.

11. The pillar was transferred to a new tube (mrk sample + E3), and points 8 and 9 were repeated.

12. The 30 eluted RNA samples were put on a freezing block immediately, and the RNA was tested by real time RT-PCR. The RNA concentration and quality/purity were measured using Thermo Scientific NanoDrop 1000 spectrophotometer version 3.8.1 with the settings "Nucleo Acids" and "DNA 40". The concentration was measured in ng/µL, good RNA quality was 260/280 ratio ~2.0 and 260/230 ratio of 2.0-2.2. The 10 best elutions with the lowest Ct-values (through real time RT-PCR) were selected for further NGS study. The RNA was kept at -80 ℃ until further use.

Appendix 9 – Nanodrop results of Trizol purification

Sample number	260/280 ratio	Sample number	260/280 ratio
B1-s100(E1)	1.14	B2-s112(E1)	1.40
B1-s100(E2)	0.99	B2-s112(E2)	1.47
B1-s100(E3)	2.94	B2-s112(E3)	1.45
B1-s108(E1)	1.40	B3-s45(E1)	1.38
B1-s108(E2)	1.24	B3-s45(E2)	1.43
B1-s108(E3)	1.73	B3-s45(E3)	1.63
B2-s69(E1)	1.44	B3-s67(E1)	1.49
B2-s69(E2)	1.47	B3-s67(E2)	1.45
B2-s69(E3)	1.46	B3-s67(E3)	1.43
B2-s105(E1)	1.52	B3-s91(E1)	1.39
B2-s105(E2)	1.32	B3-s91(E2)	1.39
B2-s105(E3)	1.18	B3-s91(E3)	1.39
B2-s111(E1)	1.06	B3-s110(E1)	1.35
B2-s111(E2)	1.34	B3-s110(E2)	1.44
B2-s111(E3)	1.37	B3-s110(E3)	1.24

*B1: Herd 1. B2: Herd 2. B3: Herd 3.

Appendix 10 – SuperScript III first strand synthesis system for RT-PCR 18080-051 (method 1)

cDNA synthesis of PRRSV: All reagents except enzymes were thawed, vortexed and spun down. The enzymes was kept on ice til just before use and then spun down.

Reaction 1:

- 1 µl RT-15392 (10 µM)
- 1 µl 10 mM dNTPs
- Total 2 µl

All reagents were put into an Eppendorf tube, mixed and afterwards put into PCR-tubes, and 8 µl RNA was added, and reaction 1 was incubated in a thermocycler at 65 ℃ in 5 minutes and afterwards cooled on an icing block for 1 minute.

Reaction 2:

- 2 µl 10x RT buffer
- 4 µl 25 mM $MgCl_2$
- 2 µl 0,1 M DTT
- 1 µl RNaseOUT
- 1 µl SuperScript III RT
- Total 10 µl

All reagents were mixed in an Eppendorf tube, and 10 µl of reaction 2 was added to reaction 1, and the reaction was set to incubate at 50 ℃ in 90 minutes and hereafter at 85 ℃ in 5 minutes. Then the sample was kept at 4 ℃. Then 1 µl RNase H was added to the reaction and it was incubated at 37 ℃ in 20 minutes and set to hold at 4 ℃ afterwards. After this step, the cDNA was produced and the sample was kept at -20 ℃ until use.

Appendix 11 – AccuPrime PCR amplification of PRRSV cDNA on products from method 1

Reaction mix 1:

- 40 µl RNase free water
- 5 µl 10x AccuPrime PCR buffer I
- 1 µl Fw primer Fragment 1-35 US fw
- 1 µl Rev primer Fragment A-US rev
- Total 48 µl

Reaction mix 2:

- 40 µl RNase free water
- 5 µl 10x AccuPrime PCR buffer I
- 1 µl Fw primer Fragment B-US-fw
- 1 µl Rev primer Fragment B-US rev
- Total 48 µl

Reaction mix 3:

- 40 µl RNase free water
- 5 µl 10x AccuPrime PCR buffer I
- 1 µl Fw primer Fragment C-US-fw
- 1 µl Rev primer Fragment C-US rev
- Total 48 µl

Reaction mix 4:

- 40 µl RNase free water
- 5 µl 10x AccuPrime PCR buffer I
- 1 µl Fw primer Fragment D-US fw
- 1 µl Rev primer Fragment RT 15392
- Total 48 µl

The different mixtures were made in 4 different Epperdorf tubes and afterwards 48 µl was placed in each PCR tube. 2 µl cDNA template was added to each reaction. Afterwards the PCR reaction was run in 6 steps shown in the following:

1. 94 ℃ in 15 seconds → 94 ℃ in 15 seconds → 50 ℃ in 30 seconds → 68 ℃ in 360 seconds #2, 44 cycles → 68 ℃ in 720 seconds → pause at 4 ℃.

PCR products were run on an agarose gel electrophoresis using E-gel® 0.8% agarose gels from Invitrogen using 10 µl, 1 kb plus ladder reference and 5 µl PCR product.

Appendix 12 – Concentration of NGS samples 1-22 and NGS samples sent to DTU

Concentration of NGS samples 1-22:

Sample	cDNA	Fragment	Sample amount from start	Optic density (ng/µl)	µl sample mixed with TE-buffer	TE-buffer
1	b1-s100	D	45µl	69.5	2.4µl	127.6µl
2	b1-s108	C	45µl	160	3.14µl	126.9µl
3	b2-s69	D	45µl	260	0.7µl	129.3µl
4	b2-s105	D	45µl	133	1.3µl	128.7µl
5	b2-s111	D	45µl	214	0.8µl	129.2µl
6	b2-s112	D	45µl	195	0.9µl	129.1µl
7	b3-s45	C	45µl	133	1.3µl	128.7µl
8	b3-s67	D	45µl	240	0.7µl	129.3µl
9	b3-s91	C	45µl	144	3.49µl	126.51µl
10	b3-s110	C	45µl	195	2.57µl	127.43µl
11	b1-s100a	Random	50µl	247	4.04µl	125.96µl
12	b1-s108a	Random	50µl	310	3.22µl	126.78µl
13	b2-s69a	Random	50µl	329	3.04µl	126.96µl
14	b2-s105a	Random	50µl	322	3.01µl	127µl
15	b2-s111a	Random	50µl	336	2.97µl	127µl

16	b2-s112a	Random	50µl	378	2.65µl	127.35µl
17	b3-s45a	Random	50µl	358	2.8µl	127.2µl
18	b3-s67a	Random	50µl	332	3.0µl	127µl
19	b3-s91a	Random	50µl	337	2.97µl	127.03µl
20	b3-s110a	Random	50µl	354	2.8µl	127.2µl
21	Porcillis	Random	50µl	338	2.9µl	127.1µl
22	Ingelvac	Random	50µl	340	2.9µl	127µl

*b1: Herd 1. b2: Herd 2. b3: Herd 3.

NGS samples sent to DTU:

Sample number	Sample ID	Size of PCR product	µg total	Concentration µg/µl	ID in CLC
15	B1-s100	2.8kb	1	0.007692308	15
16	B1-s108	7.9kb	1	0.007692308	16
17	B2-s-69	2.8kb	1	0.007692308	21
18	B2-s105	2.8kb	1	0.007692308	42
19	B2-s111	2.8kb	1	0.007692308	23
20	B2-s112	2.8kb	1	0.007692308	25
21	B3-s45	7.9kb	1	0.007692308	26
22	B3-s67	2.8kb	1	0.007692308	27
23	B3-s91	7.9kb	1	0.007692308	28

24	B3-s110	7.9kb	1	0.007692308	29
25	B1-s100a	2nd strand unknown size	1	0.007692308	30
26	B1-s108a	2nd strand unknown size	1	0.007692308	31
27	B2-s-69a	2nd strand unknown size	1	0.007692308	32
28	B2-s105a	2nd strand unknown size	1	0.007692308	33
29	B2-s111a	2nd strand unknown size	1	0.007692308	34
30	B2-s112a	2nd strand unknown size	1	0.007692308	35
31	B3-s45a	2nd strand unknown size	1	0.007692308	36
32	B3-s67a	2nd strand unknown size	1	0.007692308	37
33	B3-s91a	2nd strand unknown size	1	0.007692308	38
34	B3-s110a	2nd strand unknown size	1	0.007692308	39

Appendix 13 – Propagation of total RNA using QuantiTect Whole Transcriptome from QIAGEN (method 2)

Reaction 1 (Reverse Transcription):

- 4 µl T-Script Buffer
- 1 µl T-Script Enzyme
- ≥10 ng RNA (max 5 µl)
- Up to 10 µl RNAse-free water
- 10 µl end volume.

The sample was mixed by vortexing and spun shortly. The lid temperature was set to 50 ℃ during the whole reaction. It was incubated at 37 ℃ in 30minutes. The reaction was stopped at 95 ℃ in 5 minutes. It was cooled down to 22 ℃ (the ligation mix is added quickly).

Reaction 2 (Ligation):

- 6 µl Ligation buffer
- 2 µl Ligation Reagent
- 1 µl Enzyme 1
- 1 µl Enzyme 2
- 10 µl end volume

The sample was mixed by vortexing and spun shortly. 10 µl ligation mix was added to the tube containing reaction 1, it was mixed by vortexing and spun shortly. The mix was incubated at 22 ℃ in 2 hours (lid temperature 50 ℃).

Reaction 3 (Amplifikation):

- 29 µl REPLI-g Midi Reaction Buffer
- 1 µl REPLI-g Midi DNA polymerase
- 30 µl end volume

The sample was mixed by vortexing and spun shortly. The amplification mix was transferred to the ligase reaction. The sample was mixed by vortexing and spun shortly. The lid temperature was set to 50 ℃. The sample was incubated at 30 ℃ in 8 hours. The reaction was stopped at 95 ℃ in 5 minutes. The sample was cooled down to 4 ℃, and time was set to ∞. The cDNA was now created and it was placed at -20 ℃ for later use.

Appendix 14 – 2nd strand synthesis of PRRSV from method 2

1:

- 1 µl random primers (50 ng/µl) from SuperScript III first strand kit
- 17 µl cDNA template
- Total 18 µl

Reaction one was placed in PCR tubes and mixed and spun down and sat to incubate in a thermocycler at 95 ℃ for 2 minutes. Afterwards it was slowly cooled at room temperature.

2:

- 25.5 µl Gibco
- 5 µl 10x NEbuffer 2
- 0.5 µl dNTPs (5 U/µl) from SuperScript III first strand kit
- 1 µl Klenow Fragment (5 U/µl)
- Total 32 µl

This mix was prepared simultaneously as mix one and kept cool until use. After the two minutes, 32 µl was added to each PCR tube, and the sample was mixed and spun down and incubated in a thermocycler at 25 ℃ in 60 minutes then 75 ℃ in 10 minutes and then put on hold at 4 ℃. The PCR products were run on an agarose gel electrophoresis with an E-gel 0.8% from Invitrogen and using 10 µl, 1 kb reference 5 µl sample. This was done to show the approximate size of the fragments. The PCR-products were purified using the protocol in Roche Applied Science (Purification of PCR products by Roche Applied Science, 2016).

Appendix 15 – Productivity data

Herd 1

Sohold

Sohold til
afdeling M til M

	Periode		010716 300916	020516 300616	010416 010516	120116 310316	120116 300916
	Antal dage		92	60	31	80	263
Status	Orner ved status	Stk	7	7	7	3	7
	Søer ved status	Stk	1231	1291	1296 ?	1289 ?	1231
	Gylte ved status	Stk	322	245	180	173	322
	Polte ved status	Stk	71	92	125	114	71
	Die. grise ved status	Stk	3275	3385	3663	2905	3275
Produktion	Solgte/overførte	Stk	13482	8879	3997	11968	38326
	Producerede grise	Stk	13626	8805	4570	11260	38260
	Vægt/prod. gris	Kg	4,7	5,0	4,9	5,1	4,9
	Foder/prod. gris	FEs	49	0	0	0	17
	Sofoder/årsso	FEs	1726	0	0	0	617
	Sofoder/prod. gris	FEs	49	0	0	0	17
Årssøer	Solgte gr. /årsso+gylt	Stk	34,9	36,0	32,0	36,9	35,4
	Frav. grise/årsso+gylt	Stk	36,7	34,6	35,2	35,1	35,6
	Kuld/årsso + gylt	Stk	2,41	2,36	2,39	2,40	2,39
	Årssøer incl. gylte	Stk	1535	1500	1473	1480	1503
	Dyreenheder	DE	83	53	27	70	234
Kuldresultat	Faringer i perioden	Stk	909	559	316	778	2562
	Fravænnede kuld	Stk	885	605	272	819	2581
	Heraf 1.lægs kuld	%	17	16	16	20	18
	Levende fødte/kuld	Stk	17,5	17,4	17,4	17,1	17,4
	Sogrise/kuld	Stk	0,6	0,4	0,4	0,4	0,5
	Død fødte/kuld	Stk	1,6	1,4	1,5	1,3	1,5
	Fravænnede/kuld	Stk	15,2	14,7	14,7	14,6	14,8
	Diegivningsperiode	Dage	27	28	27	26	27
	Vægt ved fravænning	Kg	4,7	5,0	4,9	5,1	4,9
	Døde indtil fravænning	%	13,0	15,8	15,7	14,7	14,5
Reproduktion	Løbninger/Uge	Stk	74,8	80,0	67,1	68,5	73,2
	Faringer/Uge	Stk	69,2	65,2	71,4	68,1	68,2
	Fravænninger/uge	Stk	67,3	70,6	61,4	71,7	68,7
	Forventede faringer / uge	Stk	71,8	78,4	60,7	64,6	69,6
	Spildfoderdage / kuld	Dage	8	10	9	8	9
	Fra frav. til 1.løbn.	Dage	6	6	6	5	6
	Omløbere	%	1,1	2,2	2,4	1,7	1,7
	Faringsprocent	%	93,1	94,6	96,3	93,2	93,9
	Udsatte og døde dyr	Stk	245	93	44	144	526
	Døde dyr	Stk	33	22	5	28	88
	% Døde af udsatte dyr	%	13,5	23,7	11,4	19,4	16,7

Klima 30

afdeling 1 til 4

	Periode		**010716 300916**	**020516 300616**	**010416 010516**	**120116 310316**	**120116 300916**
	Antal dage		92	60	31	80	263
Status	Antal dyr ved status	Stk	7017	7699	7508	6148	7017
	Genn. vægt ved status	Kg	17,0	13,6	14,1	15,0	17,0
	Dyr ind	Stk	13482	8879	3997	11968	38326
	Dyr døde+kass.	Stk	248	130	63	188	629
	Dyr solgt	Stk	13916	8558	2574	12061	37109
Produktion	Genn. vægt ved indgang	Kg	4,7	5,0	4,9	5,1	4,9
	Genn. vægt solgte	Kg	24,3	25,3	26,0	24,5	24,7
	Genn. vægt døde+kass.	Kg	9,1	12,1	11,3	10,0	10,2
	Foderdage / dyr	Dage	42	50	73	45	47
	Døde i %	%	1,7	1,5	2,2	1,6	1,6
	Kasseret i %	%	0,0	0,0	0,0	0,0	0,0
	Staldudnyttelse	%	0	0	0	0	0
	Gennemløb / år	Stk	0,00	0,00	0,00	0,00	0,00
	Antal stipladser	Stk	0	0	0	0	0
Nøgletal	Producerede grise	Stk	14893	8497	2910	11965	38264
	Daglig tilvækst	g	467	406	290	431	421
	FEs / Kg tilvækst	FEs	1,68	0,00	0,00	0,00	0,65
	Foderstyrke FEs/dag	FEs	0,78	0,00	0,00	0,00	0,27
	Slagteprocent	%	0,0	0,0	0,0	0,0	0,0
	Kød %	%	0,0	0,0	0,0	0,0	0,0
	Dyreenheder	DE	59,2	35,0	12,4	46,9	153,5

Herd 2

Periode: 07-07-16 - 04-10-16 (90 dage)
Beregningstid 15-11-16 - 21:58:22

Status i slutningen af perioden

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Orner [#]		4	4	4	3	4
Søer [#]So [#]		589	588	565	573	589
Gilts at period end [#]		117	129	118	113	117
Polte [#]		83	71	184	91	83
Polte i første brunst [#]		5	0	0	0	5
Smågrise [#]		3.118	3.312	3.057	3.407	3.118
Pattegrise [#]		1.542	1.495	1.680	1.511	1.542

Produktionseffektivitet

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Sofoder pr årsso [FE]		1.477,7	1.461,4	1.418,3	1.427,4	1.446,6
Sofoder/ fravænnede gris [FE]		44,0	45,3	43,0	44,0	44,1
Foder pr avlsdyr pr år FE		1.268,52	1.114,82	1.075,40	1.191,79	1.159,40

Sostatistik

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Kuld / so / år [#]	2,35	2,30	2,31	2,36	2,36	2,33
Levendefødte/ so/ år		39,6	39,5	39,7	40,9	39,9
Fravænnede pattegrise / so / år [#]	33,5	34,1	33,0	32,1	33,5	33,2
Overførte smågrise / so / år [#]		33,8	28,4	33,5	28,7	31,1
Dyreenhed [DE]		44,9	47,0	46,9	43,5	182,2

Farestier per år, statistisk

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Overførte smågrise / faresti / år [#]		106	87			95
Fravænningsvægt /faresti /år [kg]		572,2	516,4			542,0

Periode: 07-07-16 - 04-10-16 (90 dage)
Beregningstid 15-11-16 - 21:58:22

Kuldresultater

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Faringer [#]	386	408	374	420	369	1.571
1 lægs kuld faringer		86	93	83	74	336
Fravænnede kuld [#]		401	388	415	384	1.588
Frav. 1. lægs søer [%]		21,4	23,5	19,5	19,0	20,8
Levende fødte pr kuld [#]	17,0	17,2	17,1	16,8	17,3	17,1
Lev. fødte på 1. lægs søer/kuld		15,7	15,2	14,1	15,8	15,2
Gns. levendevægt kg						
Gns. levendevægt på gyltekuld kg						
Levendevægt totalt kg						
Levendevægt på gyltekuld totalt kg						
Dødfødte pr kuld [#]	1,8	1,7	1,7	1,7	1,9	1,8
Fravænnede smågrise [#]	5.464	5.937	5.555	5.639	5.460	22.591
Fravænnede smågrise pr fravænning [#]	11,5	12,1	12,0	11,3	11,7	11,8
Fravænnede smågrise pr kuld [#]	14,4	14,8	14,3	13,6	14,2	14,2
Fravænningsvægt [kg]		5,42	5,23	5,36	5,58	5,40
Diegivningsperiode [days]	30	30	30	28	30	29
Regulære diegivningsdage.		27	27	25	26	26
Reele døde pattegrise døde før fravænning [%]		14,56	16,45	17,18	16,76	16,22
Beregnet pattegrisedødlighed [%]		15,0	15,5	19,6	16,7	16,7

Periode: 07-07-16 - 04-10-16 (90 dage)
Beregningstid 15-11-16 - 21:58:22

Avlsstyring

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
antal 1. brunst opmærkede polte		0	0	0	0	0
Løbninger [#]	424	447	438	436	432	1.753
Polte løbet	90	81	109	94	89	373
Løbninger per uge [#]	33	35	34	34	34	34
Faringer per uge [#]	30	32	29	33	29	31
Fravænninger per uge [#]		31	30	32	30	31
Spildfoderdage per kuld		10,4	10,7	9,5	8,1	9,7
Dage fra fravænning til 1. løbning		6,4	5,4	5,5	5,2	5,6
Omløbninger [%]		4,70	5,02	3,21	2,78	3,94
Faringsprocent [%]		91,1	92,0	93,7	91,0	92,0
Drægtighedsdage ved periodens start [dage]		31.581	29.617	32.922	29.098	29.098
Drægtighedsdage ved periodens slut [dage]		32.213	32.446	29.556	32.369	32.213
Foderdage for søer		64.549	62.902	62.372	61.430	251.253
Søer solgt [#]		80	60	79	49	268
Døde søer og gylte [#]		11	16	17	13	57
So og gylte dødelighed [%]		6,2	9,3	9,9	7,7	8,3

Effektivitetsrapport for smågrise

(Klimastald,Ungsvinstald)

Periode: 07-07-16 - 04-10-16 (90 dage)
Beregningstid 15-11-16 - 22:11:58

Status i slutningen af perioden

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Gns. vægt [kg]		14,7	14,6	0,0	0,0	14,7
Grise tilført [#]		5.937	4.818	0	0	10.755
Svin solgt/overført		5.978	3.597	859	0	10.434

Produktionseffektivitet

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Gns. vægt ved tilføjelse [kg]		5,4	5,2			5,3
Gnm. vægt, solgte smågrise [kg]						
Foderdage per gris		43,7	46,4			40,7
Døde smågrise/ prod. gris [%]		2,9	4,7			3,6

Nøgletalsindikatorer

Periode	Mål	07-07-16 - 04-10-16 90 dage	08-04-16 - 06-07-16 90 dage	09-01-16 - 07-04-16 90 dage	11-10-15 - 08-01-16 90 dage	11-10-15 - 04-10-16 360 dage
Daglig tilvækst [g]		448,0	358,5	-1.205,6		476,3
Foderudnyttelse [FE]		1,85	1,83	0,00		1,62
smågrisefoder per produceret gris FE		36,2	30,4			31,4
Dyreenhed [DE]		23,6	21,0	6,0	0,0	50,6
Alder ved salg/flytning [dage]		71,7	74,4			68,7
Alder ved 30 kg [dage]		79,1	88,3			76,2

Herd 3

Sohold

Sohold afdeling til 4 til 4

	Periode		010716 300916	010416 300616	311215 310316	011015 301215	011015 300916
	Antal dage		92	91	92	91	366
Status	Søer ved status	Stk	969	963	942	893	969
	Gylte ved status	Stk	179	196	220	239	179
	Polte ved status	Stk	130	231	219	208	130
	Frav. grise ved status	Stk	2697	3276	2661	2515	2697
	Frav. grise genn. vægt	Kg	17,8	16,5	21,2	13,8	17,8
	Die. grise ved status	Stk	2970	3287	3149	2827	2970
Produktion	Solgte/overførte	Stk	9644	8177	8971	9501	36293
	Producerede grise	Stk	9202	8391	9906	8945	36444
	Vægt/prod. gris	Kg	20,3	21,2	18,5	18,8	19,7
	Foder/prod. gris	FEs	74	68	66	71	70
	Sofoder/årsso	**FEs**	**1563**	**1241**	**1599**	**1578**	**1495**
	Foder/avlsdyr/år	FEs	1299	1041	1349	1281	1242
	Smågrisefoder/prod. gris	FEs	25	25	20	22	23
Årssøer	Prod. grise/årsso+gylt	Stk	31,7	29,1	34,6	32,5	32,0
	Frav. grise/årsso+gylt	**Stk**	**31,9**	**32,0**	**33,2**	**33,0**	**32,5**
	Kuld/årsso + gylt	**Stk**	**2,34**	**2,35**	**2,35**	**2,34**	**2,35**
	Årssøer incl. gylte	Stk	1150	1156	1137	1105	1137
Kuldresultat	Fravænnede kuld	Stk	685	662	658	624	2629
	Heraf 1.lægs kuld	%	25	26	24	23	25
	Levende fødte/kuld	**Stk**	**17,0**	**17,0**	**16,3**	**16,2**	**16,7**
	Død fødte/kuld	**Stk**	**1,9**	**1,5**	**1,3**	**1,4**	**1,5**
	Fravænnede/kuld	**Stk**	**13,6**	**13,6**	**14,1**	**14,1**	**13,9**
	Diegivningsperiode	Dage	29	28	27	27	28
	Vægt ved fravænning	Kg	6,8	6,5	6,4	6,4	6,5
	Døde indtil fravænning	**%**	**19,9**	**19,9**	**13,6**	**13,2**	**16,8**
Fravænnede grise	**Døde efter fravænning**	**%**	**2,9**	**2,8**	**1,7**	**2,6**	**2,5**
	Daglig tilvækst	**g**	**408**	**374**	**430**	**420**	**408**
	FEs / Kg tilvækst	**FEs**	**1,79**	**1,85**	**1,49**	**1,71**	**1,70**
	Foderstyrke FEs/dag	FEs	0,73	0,69	0,64	0,72	0,69
	Alder ved 30 Kg	Dage	76	81	72	73	75
Reproduktion	Løbninger/Uge	Stk	56,0	56,2	55,9	56,6	56,2
	Faringer/Uge	Stk	50,9	52,2	50,8	48,2	50,5
	Spildfoderdage / kuld	**Dage**	**9**	**10**	**10**	**10**	**10**
	Fra frav. til 1.løbn.	Dage	5	5	5	5	5
	Omløbere	**%**	**3,4**	**4,2**	**3,3**	**4,9**	**3,9**
	Faringsprocent	**%**	**92,0**	**92,3**	**90,0**	**87,5**	**90,5**
	Udsatte og døde dyr	Stk	157	147	132	156	592
	Døde dyr	Stk	26	27	24	27	104
	% Døde af udsatte dyr	%	16,6	18,4	18,2	17,3	17,6

Appendix 16 – Antibiotic use

Herd 1

Aldersgruppe 55 – pattegrise, søer, gylte, orner

ADD pr. 100 dyr pr. dag pr. antibiotikagruppe

Antibiotikagruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Penicilliner, udv.	0,22	0,37	0,21	0,30	0,21	0,40	0,14	0,20	0,33	0,43	0,14	0,28
Simple penicilliner	0,65		0,31	0,33	0,31	0,43	0,52	0,22	0,31	0,62	0,54	0,10
Sulf/trim	0,16	0,73	0,77	0,33	0,43	0,53	0,73	0,35	0,58	0,75	0,40	0,73
Ordinationsgruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Reproduktion		0,08	0,19				0,08		0,12			0,26
Tarmlidelser	0,16	0,65	0,57	0,33	0,43	0,53	0,65	0,35	0,47	0,75	0,40	0,47
Led, lemmer etc.	0,86	0,37	0,52	0,63	0,52	0,83	0,66	0,42	0,65	1,05	0,68	0,38
Gns. antal dyr/md.	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*	*1.550*
CHR gns. ADD/100 dyr/dag (9 mdr.)	*1,65*	*1,59*	*1,51*	*1,42*	*1,33*	*1,25*	*1,06*	*1,03*	*1,12*	*1,21*	*1,21*	*1,19*
Grænseværdi	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*

Aldersgruppe 56 – smågrise mellem 7-30 kg

ADD pr. 100 dyr pr. dag pr. antibiotikagruppe

Antibiotikagruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Tetracycliner	1,80	1,74	1,30	0,93		0,90	1,65	0,90		0,87	0,90	0,35
Makrolider	4,07	3,94	3,94		3,94	4,07	3,94			3,94	4,07	
Penicilliner, udv.					0,15		0,08		0,15	0,08	0,08	0,08
Pleuromutiliner	10,29	9,96	3,98	8,51	7,96	4,12	9,96	2,06	7,96	11,95	4,12	7,96
Antibiotika ej syst	1,19	3,44	2,87		2,87	2,37	2,87	2,37	1,15	3,44	2,37	2,29
Ordinationsgruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Tarmlidelser	17,34	19,08	12,10	9,44	14,77	11,46	18,42	5,33	9,11	20,20	11,46	10,61
Led, lemmer etc.					0,15		0,08		0,15	0,08	0,08	0,08
Gns. antal dyr/md.	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*	*7.500*
CHR gns. ADD/100 dyr/dag (9 mdr.)	*16,85*	*17,19*	*16,59*	*15,27*	*15,13*	*14,77*	*14,52*	*13,34*	*13,09*	*13,44*	*12,59*	*12,43*
Grænseværdi	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*

Herd 2

Aldersgruppe 55 – pattegrise, søer, gylte, orner

ADD pr. 100 dyr pr. dag pr. antibiotikagruppe

Antibiotikagruppe	Nov 2015	Dec 2015	Jan 2016	Feb 2016	Mar 2016	Apr 2016	Maj 2016	Jun 2016	Jul 2016	Aug 2016	Sep 2016	Okt 2016
Tetracycliner	0,13	0,27	0,50	0,91	0,68	0,12	0,07	0,05		0,02	0,07	0,05
Makrolider	1,24	1,20	0,96									
Lincosamider	0,02	0,05	0,05							0,02		
Penicilliner, udv.										0,48	0,17	0,58
Simple penicilliner	1,44	1,63	1,10	1,49	1,10	1,34	0,96	0,74	0,72	1,25	0,35	0,96
Sulf/trim	0,15			0,11	0,25	0,30				0,07		
Streptomycinpræparater	0,35	0,29	0,24	0,31	0,29	0,15	0,10	0,45	0,05			
Amfenikoler		0,24			0,10		0,10	0,15		0,14	0,10	
Ordinationsgruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Reproduktion							0,86	0,60	0,72	1,03	0,35	0,96
Yver	1,39	1,54	0,96	1,40	1,35	1,54						
Tarmlidelser	0,02	0,29			0,10		0,10	0,15		0,14	0,10	
Luftvejslidelser	1,24	1,20	1,01	0,86	0,51	0,08	0,05					
Led, lemmer etc.	0,68	0,65	0,89	0,57	0,46	0,29	0,22	0,64	0,05	0,82	0,24	0,62
Gns. antal dyr/md.	*672*	*672*	*672*	*672*	*672*	*672*	*672*	*672*	*672*	*672*	*672*	*672*
CHR gns. ADD/100 dyr/dag (9 mdr.)	*2,89*	*2,83*	*2,91*	*2,96*	*3,02*	*2,89*	*2,68*	*2,55*	*2,26*	*2,12*	*1,78*	*1,64*
Grænseværdi	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*

Aldersgruppe 56 – smågrise mellem 7-30 kg

ADD pr. 100 dyr pr. dag pr. antibiotikagruppe

Antibiotikagruppe	Nov 2015	Dec 2015	Jan 2016	Feb 2016	Mar 2016	Apr 2016	Maj 2016	Jun 2016	Jul 2016	Aug 2016	Sep 2016	Okt 2016
Tetracycliner	9,48	6,19	6,03	11,47	19,78	6,96	9,46	6,59	3,02	12,27	9,35	6,17
Makrolider	0,65	0,95	0,32	1,01		4,92						
Lincosamider				0,07								
Penicilliner, udv.							12,06					12,06
Simple penicilliner					0,14						0,29	
Streptomycinpræparater	0,57	0,28		0,89	0,14		0,97	1,29	0,14			0,69
Ordinationsgruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Tarmlidelser	9,48	6,19	6,03	9,67	18,10	11,66	9,26	6,45	3,02	12,20	9,35	18,24
Luftvejslidelser	0,65	0,95	0,32	2,81	1,68	0,22	12,27	0,14		0,07		
Led, lemmer etc.	0,57	0,28		0,96	0,28		0,97	1,29	0,14		0,29	0,69
Gns. antal dyr/md.	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*	*3.100*
CHR gns. ADD/100 dyr/dag (9 mdr.)	*11,94*	*10,67*	*10,62*	*11,25*	*11,52*	*11,42*	*12,17*	*11,93*	*11,49*	*11,66*	*11,92*	*13,34*
Grænseværdi	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*

Herd 3

Aldersgruppe 55 – pattegrise, søer, gylte, orner

ADD pr. 100 dyr pr. dag pr. antibiotikagruppe

Antibiotikagruppe	Nov 2015	Dec 2015	Jan 2016	Feb 2016	Mar 2016	Apr 2016	Maj 2016	Jun 2016	Jul 2016	Aug 2016	Sep 2016	Okt 2016
Tetracycliner	0,06	0,08	0,05				0,08			0,09	0,10	
Makrolider	0,36	0,49	0,35	0,30								
Lincosamider										0,05		0,05
Penicilliner, udv.	0,21	0,20	0,08	0,22	0,61		0,31	0,42	0,10	0,20	0,32	
Simple penicilliner	0,25	0,22	0,09	0,79	0,92		1,23	1,05	0,37	0,18	0,95	0,74
Sulf/trim	1,32	1,46	1,94	1,41	1,50		2,03	1,46	1,57	1,64	0,99	2,49
Streptomycinpræparater									0,46		0,19	0,06
Antibiotika ej syst							0,31			0,31	0,32	0,31
Ordinationsgruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Reproduktion								1,55	1,57	1,64	1,18	2,49
Yver	1,44	1,46	1,97	1,61	1,69		2,18					
Tarmlidelser							0,31			0,36	0,32	0,36
Luftvejslidelser	0,36	0,49	0,35	0,30								
Led, lemmer etc.	0,40	0,50	0,20	0,81	1,35		1,46	1,38	0,93	0,48	1,37	0,80
Gns. antal dyr/md.	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*	*1.050*
CHR gns. ADD/100 dyr/dag (9 mdr.)	*2,33*	*2,32*	*2,48*	*2,38*	*2,50*	*2,30*	*2,48*	*2,38*	*2,48*	*2,52*	*2,56*	*2,69*
Grænseværdi	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*	*4,30*

Aldersgruppe 56 – smågrise mellem 7-30 kg

ADD pr. 100 dyr pr. dag pr. antibiotikagruppe

Antibiotikagruppe	Nov 2015	Dec 2015	Jan 2016	Feb 2016	Mar 2016	Apr 2016	Maj 2016	Jun 2016	Jul 2016	Aug 2016	Sep 2016	Okt 2016
Tetracycliner	6,71			5,00	7,98		10,51	5,56				
Makrolider	1,75		0,49	0,52			0,49	3,67	2,15	2,21	3,67	5,03
Penicilliner, udv.	1,85		0,79	1,53	1,43		0,72	2,59		0,43	0,44	0,36
Simple penicilliner	1,54	1,27	1,61	2,07	1,94						0,67	
Pleuromutiliner	10,72		14,93		5,38		8,06		8,06	8,06	5,56	5,38
Ordinationsgruppe	**Nov 2015**	**Dec 2015**	**Jan 2016**	**Feb 2016**	**Mar 2016**	**Apr 2016**	**Maj 2016**	**Jun 2016**	**Jul 2016**	**Aug 2016**	**Sep 2016**	**Okt 2016**
Tarmlidelser	17,43		14,93	5,00	13,36		18,57	5,56	8,06	8,06	7,84	9,06
Luftvejslidelser	3,30	1,27	2,10	2,59	1,94		0,49	3,67	2,15	2,21	2,06	1,34
Led, lemmer etc.	1,85		0,79	1,53	1,43		0,72	2,59		0,43	0,44	0,36
Gns. antal dyr/md.	*2.880*	*3.386*	*4.000*	*4.000*	*4.000*	*4.000*	*4.000*	*4.000*	*4.000*	*4.000*	*4.000*	*4.000*
CHR gns. ADD/100 dyr/dag (9 mdr.)	*16,88*	*15,35*	*15,65*	*14,27*	*13,21*	*11,66*	*12,77*	*12,34*	*12,04*	*11,05*	*11,91*	*11,11*
Grænseværdi	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*	*22,90*

Appendix 17 – Clinical registrations

Herd 1

Total number of pigs	Unthrifty (anorexic/longhaired)	Respiratory distress (sneezing, coughing, dys- and hyperpnea)	Diarrhea Blobs per pen
Week 4 **970 pigs**	IIIII IIIII IIIII IIII = 19 pigs Prevalence: 19 pigs / 970 pigs = 1,96 %	IIIII IIIII IIIII IIIII IIIII IIIII III = 33 pigs Prevalence: 33 pigs / 970 pigs = 3,4 %	4 blobs of diarrhea in a total of 18 pens
Week 8 **647 pigs**		IIIII IIIII I = 11 pigs Prevalence: 11 pigs / 647 pigs = 1,7 %	2 blobs of diarrhea in a total of 18 pens
Week 12 **477 pigs**		IIII = 4 pigs Prevalence: 4 pigs / 477 pigs = 0,8 %	5 blobs of diarrhea in a total of 18 pens

Herd 2

Total number of pigs	Unthrifty (anorexic/longhaired)	Respiratory distress (sneezing, coughing, dys- and hyperpnea)	Diarrhea Blobs per pen
Week 4 **172 pigs**		III = 3 pigs *Prevalence:* 3 pigs /172 pigs = 1,7 %	1 blobs of diarrhea in a total of 10 pens
Week 8 **223 pigs**	III = 3 pigs *Prevalence:* 3 pigs /223 pigs= 1,3%	IIIII IIIII = 10 pigs *Prevalence:* 10 pigs / 223 pigs = 4,5 %	5 blobs of diarrhea in a total of 6 pens
Week 12 **334 pigs**	III = 3 pigs *Prevalence:* 3 pigs/334 pigs = 0,9 %	IIIII IIIII IIIII = 15 pigs *Prevalence:* 15 pigs / 334 pigs = 4,5 %	3 blobs of diarrhea in a total of 11 pens

Herd 3

Total number of pigs	**Unthrifty (anorexic/longhaired)**	**Respiratory distress (sneezing, coughing, dys- and hyperpnea)**	**Diarrhea Blobs per pen**
Week 4 **678 pigs**	IIIII IIIII IIIII IIII = 19 pigs *Prevalence:* 19 pigs /678 pigs= 2,8%	There could be heard sneezing all the time Furthermore there was observed serous mucos from more pigs *Coughing:* IIIII III = 8 pigs *Prevalence (coughing):* 8 pigs / 678 pigs = 1,2%	3 blobs of diarrhea in a total of 12 pens
Week 8 **515 pigs**	IIIII IIIII = 10 pigs *Prevalence:* 10 pigs /515 pigs= 1,9%	IIIII IIIII I = 11 pigs *Prevalence:* 11 pigs / 515 pigs = 2,1 %	
Week 12 **287 pigs**		IIII = 4 pigs *Prevalence:* 4 pigs / 287 pigs = 1,4 %	

Appendix 18 – Results from PCR screening

Herd 1			
Pooled sample number	Kind of sample	Ct-value	Rep-Ct value
13	serum 12 weeks	33.9	33.64
		33.37	
14	serum 12 weeks	30.97	30.97
		30.05	
15	serum 12 weeks	31.19	31.45
		31.72	
16	serum 12 weeks	27.46	27.53
		27.6	
18	serum 12 weeks	30.06	30.29
		30.52	
13	tonsil swab 12 weeks	36.12	35.86
		35.6	
14	tonsil swab 12 weeks	33.66	33.65
		33.65	
15	tonsil swab 12 weeks	36.17	36.37
		36.56	
16	tonsil swab 12 weeks	36.48	35.88

		35.28	
18	tonsil swab 12 weeks	38.48	37.49
		36.5	

Herd 2			
Pooled sample number	Kind of sample	Ct-value	Rep-Ct value
7	serum 8 weeks	32.67	34.57
		36.46	
8	serum 8 weeks	26.64	26.79
		26.93	
9	serum 8 weeks	34.26	36.12
		37.99	
10	serum 8 weeks	30.66	31.08
		31.51	
13	serum 12 weeks	35.29	34.4
		33.5	
14	serum 12 weeks	37.64	36.85
		36.06	
15	serum 12 weeks	29.16	29.36

		29.55	
16	serum 12 weeks	30.83	31.1
		31.37	
17	serum 12 weeks	27.51	27.55
		27.6	
18	serum 12 weeks	35.13	34.55
		33.98	
13	tonsil swab 12 weeks	35.72	35.7
		35.69	
14	tonsil swab 12 weeks	37.12	37.01
		36.91	
15	tonsil swab 12 weeks	35.72	36.45
		37.18	
16	tonsil swab 12 weeks	40.12	38.28
		36.43	
17	tonsil swab 12 weeks	36.85	36.23
		35.6	
18	tonsil swab 12 weeks	35.77	35.24
		34.71	

Herd 3			
Pooled sample number	Kind of sample	Ct-value	Rep-Ct value
2	serum 4 weeks	37.35	36.9
		36.44	
3	serum 4 weeks	37.82	37.65
		37.47	
5	serum 4 weeks	31.46	31.45
		31.43	
8	serum 8 weeks	31.84	33.31
		33.77	
9	serum 8 weeks	35.14	35.32
		35.5	
13	serum 12 weeks	26.46	26.57
		26.67	
14	serum 12 weeks	31.99	32.06
		32.13	
15	serum 12 weeks	30.25	30.15
		30.06	
16	serum 12 weeks	25.95	25.62

		25.29	
17	serum 12 weeks	38.46	
18	serum 12 weeks	36.07	37.4
		38.73	
13	tonsil swab 12 weeks	33.52	33.31
		33.1	
14	tonsil swab 12 weeks	31.48	32.45
		33.41	
15	tonsil swab 12 weeks	32.47	32.54
		32.61	
16	tonsil swab 12 weeks	31.14	31.27
		31.4	
17	tonsil swab 12 weeks	37.21	37.14
		37.06	
18	tonsil swab 12 weeks	32.16	32.15
		32.14	

Appendix 19 – Samples selected for single sample purification

Herd	Sample	Age	Rep-Ct value
1	14	serum, 12 weeks	30.97
1	16	serum, 12 weeks	27.53
1	14	tonsil swab, 12 weeks	33.65
1	16	tonsil swab, 12 weeks	35.88
2	8	serum, 8 weeks	26.79
2	15	serum, 12 weeks	29.36
2	17	serum, 12 weeks	27.55
2	17	tonsil swab, 12 weeks	36.23
3	5	serum, 4 weeks	31.45
3	8	serum, 8 weeks	33.31
3	13	serum, 12 weeks	26.57
3	16	serum, 12 weeks	25.62
3	16	tonsil swab, 12 weeks	31.27

Appendix 20 – Single samples from each pool screened using real time RT-PCR

Herd 1			
Sample	Age of the pig	Ct-value	Rep-Ct value
s-96	serum, 12 weeks	34.34	34.94
		35.55	
s-97	serum, 12 weeks	35.17	34.75
		34.33	
s-98	serum, 12 weeks	33.56	33.46
		33.37	
s-99	serum, 12 weeks	32.25	31.85
		31.45	
s-100	serum, 12 weeks	29.46	29.1
		28.74	
s-108	serum, 12 weeks	28.95	29.06
		29.16	
s-109	serum, 12 weeks	33.75	33.8
		33.87	
s-110	serum, 12 weeks	37.98	38.51
		39.05	

t-96	tonsil swab, 12 weeks	34.38	34.25
		34.11	
t-97	tonsil swab, 12 weeks	32.95	33.82
		34.68	
t-98	tonsil swab, 12 weeks	34.32	34.02
		33.72	
t-99	tonsil swab, 12 weeks	35.24	34.79
		34.33	
t-100	tonsil swab, 12 weeks	34.16	34.32
		34.49	
t-108	tonsil swab, 12 weeks	33.16	34.1
		35.04	

Herd 2			
Sample	Age of the pig	Ct-value	Rep-Ct value
s-69	serum, 8 weeks	27.29	27.2
		27.12	
s-101	serum, 12 weeks	36.81	36.82
		36.83	
s-102	serum, 12 weeks	32.67	32.52

		32.36	
s-103	serum, 12 weeks	37.12	36.94
		36.76	
s-104	serum, 12 weeks	35.02	34.98
		34.94	
s-105	serum, 12 weeks	29.87	30.35
		30.84	
s-111-17	serum, 12 weeks	29.45	29.51
		29.57	
s-112	serum, 12 weeks	25.98	25.55
		25.12	
s-113	serum, 12 weeks	36.9	37.44
		37.98	
s-115	serum, 12 weeks	32.35	32.55
		32.74	
t-111	tonsil swab, 12 weeks	34.77	34.51
		34.25	
t-112	tonsil swab, 12 weeks	33.12	32.86
		32.6	

t-115	tonsil swab, 12 weeks	33.7	33.79
		33.88	

Herd 3			
Sample	Age of the pig	Ct-value	Rep-Ct value
s-42	serum, 4 weeks	31.6	31.55
		31.5	
s-45	serum, 4 weeks	27.31	27.57
		27.84	
s-67	serum, 8 weeks	27.85	27.86
		27.86	
s-91	serum, 12 weeks	24.41	24.45
		24.5	
s-92	serum, 12 weeks	33.92	34.01
		34.11	
s-93	serum, 12 weeks	36.21	36.11
		36	
s-94	serum, 12 weeks	34.47	34.61
		34.75	
s-95	serum, 12 weeks	25.14	24.97

		24.8	
s-106	serum, 12 weeks	26.85	26.76
		26.67	
s-107	serum, 12 weeks	34.06	34.09
		34.11	
s-108	serum, 12 weeks	25.1	25.4
		25.7	
s-109	serum, 12 weeks	26.83	26.83
s-110	serum, 12 weeks	25.48	24.89
		24.3	
t-106	tonsil swab, 12 weeks	35.73	35.23
		34.74	
t-107	tonsil swab, 12 weeks	37.04	36.49
		35.95	
t-108	tonsil swab, 12 weeks	32.12	32.61
		33.1	
t-109	tonsil swab, 12 weeks	29.71	29.51
		29.31	

t-110	tonsil swab, 12 weeks	34.04	34.27
		34.51	

Appendix 21 – Samples selected for Sanger sequencing and NGS

19 samples were selected for Sanger sequencing. From these 19, 10 samples were selected for Next-Generation Sequencing.

Herd 1		
Sample	Age of the pig	Rep-Ct value
s99	12 weeks	31.85
s100(NGS)	12 weeks	29.1
s108(NGS)	12 weeks	29.06
t100	12 weeks	34.32
t108	12 weeks	34.1

Herd 2		
Sample	Age of the pig	Rep-Ct value
s69(NGS)	8 weeks	27.2
s105(NGS)	12 weeks	30.35
s111(NGS)	12 weeks	29.51
s112(NGS)	12 weeks	25.55
t111	12 weeks	34.51
t112	12 weeks	32.86

Herd 3		
Sample	Age of the pig	Rep-Ct value
s42	4 weeks	31.55
s45(NGS)	4 weeks	27.57
s67(NGS)	8 weeks	27.86
s91(NGS)	12 weeks	24.45
s95	12 weeks	24.97
s108	12 weeks	25.4
s110(NGS)	12 weeks	24.89
t109	12 weeks	29.51

Appendix 22 – Results from Sanger sequencing of PRRSV ORF5 US

Sample number	Sample name	RNA obtained (date)	E-gel result (bp length)
1	156(b1-s99)	156 – 17/11-16	818 bp (US)
2	157(b1-s100)	157 - 17/11-16	818 bp (US)
3	160(b1-s108)	160– 17/11-16	818 bp (US)
4	167(b1-t100)	167– 17/11-16	818 bp (US)
5	170(b1-t108)	170– 17/11-16	818 bp (US)
6	177(b2-s69)	177– 17/11-16	818 bp (US)
7	185(b2-s105)	185– 17/11-16	818 bp (US)
8	186(b2-s111)	186– 17/11-16	818 bp (US)
9	187(b2-s112)	187– 17/11-16	818 bp (US)
10	191(b2-t111)	191– 17/11-16	818 bp (US)
11	192(b2-t112)	192– 17/11-16	818 bp (US)
12	197(b3-s42)	197– 17/11-16	NEG
13	202(b3-s45)	202– 17/11-16	818 bp (US)
14	209(b3-s67)	209– 17/11-16	818 bp (US)
15	213(b3-s91)	213– 17/11-16	818 bp (US)
16	217(b3-s95)	217– 17/11-16	818 bp (US)
17	220(b3-s108)	220– 17/11-16	818 bp (US)
18	222(b3-s110)	222– 17/11-16	818 bp (US)

19	228(b3-t109)	228– 17/11-16	818 bp (US)
20	Pos Control - Ingelvac		818 bp (US)

*b1: Herd 1. b2: Herd 2. b3: Herd 3.

Appendix 23 – PRRSV type 2 ORF5 sequences used for phylogenetic tree

No.	Sequence ID	Description	Basepair length
1	AY875854	Porcine reproductive and respiratory syndrome virus strain 2889 envelope glycoprotein gene, complete cds	603
2	AY615794.1	Porcine respiratory and reproductive syndrome virus strain LU534 envelope glycoprotein (ORF5) gene, complete cds	603
3	AY615795.1	Porcine respiratory and reproductive syndrome virus strain SE521 envelope glycoprotein (ORF5) gene, complete cds	603
4	U64929.1	Porcine reproductive and respiratory syndrome virus strain IAF-BAJ envelope glycoprotein (E), matrix protein (M), and nucle-ocapsid protein (N) genes, complete cds	1563
5	AH006184.2	Porcine reproductive and respiratory syndrome virus strain PA-8 minor membrane glycoprotein, glycoprotein, major membrane glycoprotein, membrane protein, and nucleocapsid protein genes, complete cds	2961
6	U64928.1	Porcine reproductive and respiratory syndrome virus strain IAF-Klop envelope glycoprotein (E), matrix protein (M), and nucle-ocapsid protein (N) genes, complete cds	1563
7	GQ374442.1	Porcine reproductive and respiratory syndrome virus strain GDBY1, complete genome	15338
8	GQ374441.1	Porcine reproductive and respiratory syndrome virus strain GDQJ, complete genome	15339
9	AY032626.1	Porcine reproductive and respiratory syndrome virus strain CH-	15432

		1a, complete genome	
10	AY881994.1	Porcine reproductive and respiratory syndrome virus strain FJ-1 GP2 envelope glycoprotein, unknown protein, GP3 envelope protein, GP4 envelope glycoprotein, GP5 glycosylated envelope protein, membrane protein M, and nucleocapsid protein N genes, complete cds	3188
11	EF112445.1	Porcine respiratory and reproductive syndrome virus strain JXA1, complete genome	15347
12	KC862576.1	Porcine reproductive and respiratory syndrome virus isolate DK-1997-19407B, complete genome	15399
13	KC506665.1	Porcine reproductive and respiratory syndrome virus isolate DK-2003-1-2 glycoprotein 5 (ORF5) mRNA, complete cds	603
14	KC862584.1	Porcine reproductive and respiratory syndrome virus isolate DK-2003-2-3, complete genome	15411
15	KC506667.1	Porcine reproductive and respiratory syndrome virus isolate DK-2003-3-3 glycoprotein 5 (ORF5) mRNA, complete cds	603
16	KC506668.1	Porcine reproductive and respiratory syndrome virus isolate DK-2003-4-1 glycoprotein 5 (ORF5) mRNA, complete cds	603
17	KC506669.1	Porcine reproductive and respiratory syndrome virus isolate DK-2003-5-1 glycoprotein 5 (ORF5) mRNA, complete cds	603
18	KC862578.1	Porcine reproductive and respiratory syndrome virus isolate DK-2004-1-7-Pl, complete genome	15411
19	KC862585.1	Porcine reproductive and respiratory syndrome virus isolate DK-2004-2-1, complete genome	15411

20	KC577601.1	Porcine reproductive and respiratory syndrome virus isolate DK-2004-3-1 glycoprotein 5 (ORF5) gene, complete cds	603
21	KC862582.1	Porcine reproductive and respiratory syndrome virus isolate DK-2008-10-1-3, complete genome	15411
22	KC506671.1	Porcine reproductive and respiratory syndrome virus isolate DK-2008-16-2-4 glycoprotein 5 (ORF5) mRNA, complete cds	603
23	KC506672.1	Porcine reproductive and respiratory syndrome virus isolate DK-2008-16-3-3 glycoprotein 5 (ORF5) mRNA, complete cds	603
24	KC862579.1	Porcine reproductive and respiratory syndrome virus isolate DK-2010-10-1-2, complete genome	15345
25	KC506628.1	Porcine reproductive and respiratory syndrome virus isolate DK-2010-10-2-2 glycoprotein 5 (ORF5) mRNA, complete cds	603
26	KC506630.1	Porcine reproductive and respiratory syndrome virus isolate DK-2010-10-3-3 glycoprotein 5 (ORF5) mRNA, complete cds	603
27	KC506633.1	Porcine reproductive and respiratory syndrome virus isolate DK-2010-10-4-3 glycoprotein 5 (ORF5) mRNA, complete cds	603
28	KC506634.1	Porcine reproductive and respiratory syndrome virus isolate DK-2010-10-5-2 glycoprotein 5 (ORF5) mRNA, complete cds	603
29	KC506635.1	Porcine reproductive and respiratory syndrome virus isolate DK-2010-10-6-3 glycoprotein 5 (ORF5) mRNA, complete cds	603
30	KC862580.1	Porcine reproductive and respiratory syndrome virus isolate DK-2010-10-7-1, complete genome	15402
31	KC506637.1	Porcine reproductive and respiratory syndrome virus isolate DK-	603

		2010-30-8-4 glycoprotein 5 (ORF5) mRNA, complete cds	
32	KC506645.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-030311-4 glycoprotein 5 (ORF5) mRNA, complete cds	603
33	KC506641.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-10-2-1 glycoprotein 5 (ORF5) mRNA, complete cds	603
34	KC506652.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-10-5-1 glycoprotein 5 (ORF5) mRNA, complete cds	603
35	KC506639.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-30-1-34 glycoprotein 5 (ORF5) mRNA, complete cds	603
36	KC506648.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-30-3-20 glycoprotein 5 (ORF5) mRNA, complete cds	600
37	KC506649.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-30-4-3 glycoprotein 5 (ORF5) mRNA, complete cds	603
38	KC506651.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-30-4-7 glycoprotein 5 (ORF5) mRNA, complete cds	603
39	KC506653.1	Porcine reproductive and respiratory syndrome virus isolate DK-2011-30-6-27 glycoprotein 5 (ORF5) mRNA, complete cds	603
40	KC862575.1	Porcine reproductive and respiratory syndrome virus isolate DK-2012-01-11-3, complete genome	15402
41	KC506654.1	Porcine reproductive and respiratory syndrome virus isolate DK-2012-10-1-5 glycoprotein 5 (ORF5) mRNA, complete cds	603
42	KC506655.1	Porcine reproductive and respiratory syndrome virus isolate DK-2012-10-2-8 glycoprotein 5 (ORF5) mRNA, complete cds	603

43	KC506657.1	Porcine reproductive and respiratory syndrome virus isolate DK-2012-10-3-1 glycoprotein 5 (ORF5) mRNA, complete cds	603
44	KC506659.1	Porcine reproductive and respiratory syndrome virus isolate DK-2012-30-7-18 glycoprotein 5 (ORF5) mRNA, complete cds	603
45	DK-2015-7-4	From Lise Kvisgaard	603
46	DK-2015-8-32	From Lise Kvisgaard	603
47	DK.2015-9-24	From Lise Kvisgaard	603
48	DK-2015-6-18	From Lise Kvisgaard	603
49	KC714034.1	Porcine reproductive and respiratory syndrome virus strain SP1 major envelope glycoprotein GP5 gene, complete cds	603
50	DQ475799.1	Porcine respiratory and reproductive syndrome virus isolate PRRSV0001298 envelope glycoprotein gene, complete cds	603
51	JN651746.1	Porcine reproductive and respiratory syndrome virus strain BH_95/10-08_NA major envelope glycoprotein GP5 gene, complete cds	603
52	KC714024.1	Porcine reproductive and respiratory syndrome virus strain 660501 major envelope glycoprotein GP5 gene, partial cds	600
53	KC714023.1	Porcine reproductive and respiratory syndrome virus strain 659551 major envelope glycoprotein GP5 gene, partial cds	600

54	EF484033.1	Porcine respiratory and reproductive syndrome virus clone pMLV, complete genome	15412
55	D45852.1	Porcine respiratory and reproductive syndrome virus genes for glycoprotein, membrane protein, nucleocapsid protein, complete cds	1713
56	AB175720.1	Porcine respiratory and reproductive syndrome virus gene for envelope glycoprotein GP5, complete cds	603
57	AB175713.1	Porcine respiratory and reproductive syndrome virus gene for envelope glycoprotein GP5, complete cds	603
58	JF681207.1	Porcine reproductive and respiratory syndrome virus isolate J14-12 glycoprotein 5 mRNA, complete cds	603
59	FJ972737.1	Porcine reproductive and respiratory syndrome virus isolate 4033-12-V-2008 envelope protein mRNA, complete cds	603
60	M96262.2	Lelystad virus, complete genome	15111
61	KC714027.1	Porcine reproductive and respiratory syndrome virus strain Jar-72 major envelope glycoprotein GP5 gene, complete cds	603
62	KC714018.1	Porcine reproductive and respiratory syndrome virus strain 11_01105 major envelope glycoprotein GP5 gene, complete cds	603
63	KC714019.1	Porcine reproductive and respiratory syndrome virus strain 11_01109 major envelope glycoprotein GP5 gene, complete cds	603
64	KC714031.1	Porcine reproductive and respiratory syndrome virus strain RJ_J_T major envelope glycoprotein GP5 gene, complete cds	603
65	AY297118.1	Porcine reproductive and respiratory syndrome virus strain 02SP3	600

		GP5 envelope protein (ORF5) gene, complete cds	
66	EF532816.1	Porcine reproductive and respiratory syndrome virus strain ISU-P, complete genome	15193
67	AF046869.1	Porcine reproductive and respiratory syndrome virus isolate 16244B, 2/18/97(Nebraska)pass.3, complete genome	15428
68	DQ306877.1	Porcine respiratory and reproductive syndrome virus isolate 98-3298 glycoprotein 5 gene, complete cds	603
70	DQ176019.1	Porcine respiratory and reproductive syndrome virus isolate MN184A, complete genome	15019
71	DQ176020.1	Porcine respiratory and reproductive syndrome virus isolate MN184B, complete genome	15019
73	DQ476536.1	Porcine respiratory and reproductive syndrome virus isolate PRRSV0002239 envelope glycoprotein gene, complete cds	603
74	DQ478403.1	Porcine respiratory and reproductive syndrome virus isolate PRRSV0004557 envelope glycoprotein gene, complete cds	603
75	HQ699067.1	Porcine reproductive and respiratory syndrome virus strain NC16845, complete genome	15389
76	EU758863.1	Porcine respiratory and reproductive syndrome virus isolate PRRSV0000008880 envelope glycoprotein gene, complete cds	603
77	U34296.1	Porcine reproductive and respiratory syndrome virus strain ISU1894 ORF2, ORF3, ORF4 and putative envelope protein genes, complete cds	2318
78	U34300.1	Porcine reproductive and respiratory syndrome virus strain ISU79	2318

		ORF2, ORF3, ORF4 putative envelope protein genes, complete cds	
79	JX044140.1	Porcine reproductive and respiratory syndrome virus strain VR2385, complete genome	15002