Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

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Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

Technical University of Denmark

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PhD thesis, July 2013

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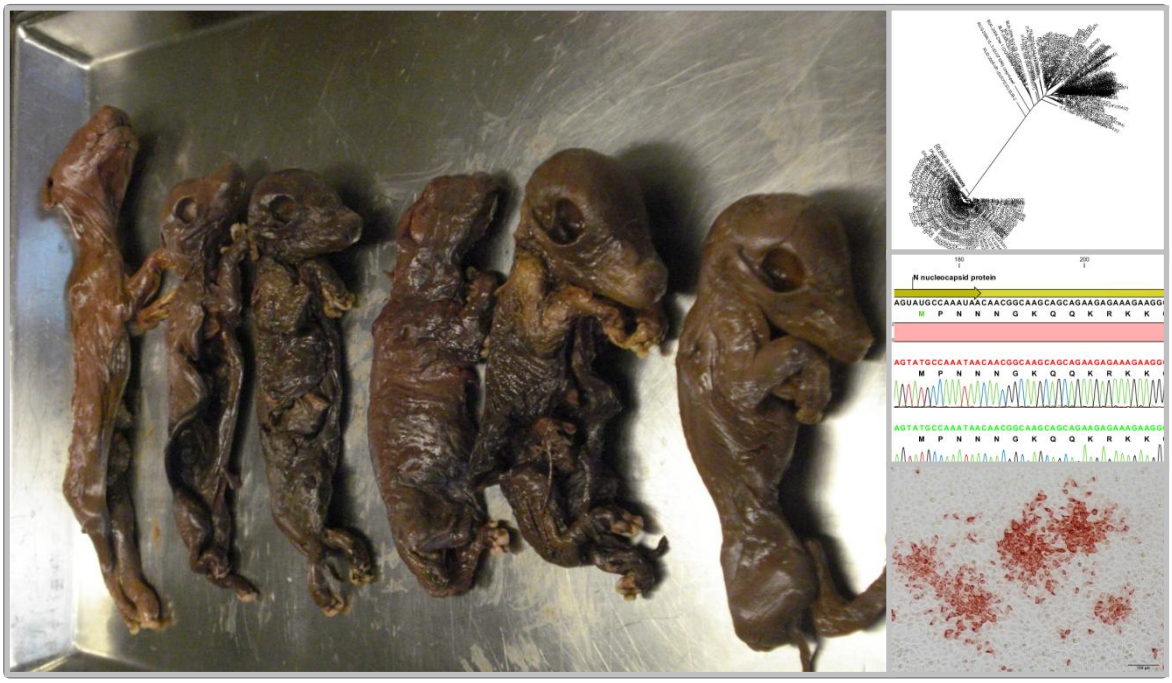

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TABLE OF CONTENTS

PREFACE

The majority of the experimental work on which this PhD thesis is based, has been performed at the Technical University of Denmark (DTU), National Veterinary Institute, Frederiksberg from July 2010 to June 2013.

I also had the opportunity to go abroad for 6 month, to visit The Roslin Institute, University of Edinburgh, Scotland, UK from January to June 2012.

The work of this PhD regarding the diversity studies was partly funded from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 245141 (New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia (PoRRSCon) coordinated by Prof. H. Nauwynck) and the COST Action FA902: Understanding and combating porcine reproductive and respiratory syndrome in Europe (EuroPRRS.net).

The experimental infection study performed at the Technical University of Denmark (DTU), National Veterinary Institute, Lindholm was kindly funded by Boehringer Ingelheim Animal Health, Germany.

I would like to express my gratitude to the following people who all contributed to the studies and the thesis in various ways:

First of all, I wish to thank my supervisor Professor Lars E. Larsen for giving me the opportunity to become a PhD. I am grateful for your help and guidance throughout the three years in your laboratory and for your scientific inputs and for patiently answering all my questions.

Grateful thanks go to co-supervisor Dr. Charlotte K. Hjulsager for your every-day help with my project. Our afternoon discussions have been priceless, and you have had a huge impact on my motivation and the outcome of this PhD project.

I am grateful to Dr. Tahar Ait-Ali for giving me the opportunity to have a research stay at the Roslin Institute and for taking good care of me during my stay abroad. Furthermore, I would like to thank all the people I met during my stay in Scotland, especially Natalie Lowe and Heather Finlayson for their kind assistance in the laboratory.

For technical assistance in the laboratory I would like to thank all the technicians from the virology group, especially Hue Thi Thanh Tran for her great work on PRRSV, I know it can be a struggle at times.

I would like to thank all the people at Lindholm whom I have collaborated with during my PhD. Thank you Thomas Bruun Rasmussen for providing the protocols for full genome cDNA synthesis and long range PCR amplification.

Special thanks to Dr. Jens Nielsen and Dr. Anette Bøtner for their work regarding the experimental infection study and to laboratory technician Janne Holm Hansen for her help and expertise in culturing and handling of PRRSV. I would also like to thank Mette Sif Hansen for performing the autopsy of the pigs and Peter Heegaard for performing the Acute Phase protein measurements.

Thanks to Dr. Klara Tølbøll Lauritsen for providing serum samples, Charlotte Sonne Kristensen and PH Rathkjen for providing field samples, and to Dr. Ramona Trebbien and Dr. Solvej Ø. Breum for their always kind help and guidance.

Finally, I would like to thank my fellow PhD students, Kristina Fobian and Jesper Schak Krog, for their good companionship at the office. We have had some good laughs!

Frederiksberg, July 2013

Lise Kirstine Kvisgaard

ABBREVIATIONS

ATCC	American Type Culture Collection
CPE	Cytopathic effect
Ct	Cycle threshold
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphates
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
E	Envelope protein
EAV	Equine Arteritis Virus
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
GAGs	Glycosaminoglycans
GP	Glycoprotein
IFN	Interferon
IPMA	Immunoperoxidase monolayer assay
Kb	Kilobase
LDV	Lactate Dehydrogenase Elevation Virus
LV	Lelystad Virus
M	Membrane protein
MEM	Minimum essential medium
MQ	Milli Q water
N	Nucleocapsid protein
NEAA	Non-essential amino acids
NGS	Next generation sequencing
NSP	Non-structural protein (coding region)
nsp	Non-structural protein (protein product)
OD	Optical density
ORF	Open reading frame
PAM	Porcine alveolar macrophages

PCPα	Papain-like cysteine protease domain α
PCPβ	Papain-like cysteine protease domain β
PCR	Polymerase chain reaction
Pen/strep	Penicillin/Streptomycin
PI	Post infection
pp1a	Polyprotein 1a
pp1ab	Polyprotein 1ab
PriProET	Primer Probe Energy Transfer
PRRS	Porcine Reproductive and Respiratory Syndrome
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RACE	Rapid amplification of cDNA ends
RdRp	RNA-dependent RNA polymerase
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
sg	Subgenomic
SHFV	Simian Hemorrhagic Fever Virus
SP	Serine protease
Sn	Sialoadhesin
ssDNA	Single-stranded DNA
TdT	Terminal deoxynucleotidyl transferase
UTR	Untranslated region
VR-2332	ATCC VR-2332

SUMMARY

This PhD thesis presents the diversity of Porcine Reproductive and Respiratory Syndrome viruses (PRRSV) circulating in the Danish pig population. PRRS is a disease in pigs caused by the PRRS virus resulting in reproductive failures in sows and gilts and respiratory diseases in pigs. Due to genetic heterogeneity, PRRSV is divided into two genotypes, Type 1 and Type 2. Type 1 PRRS viruses are further divided into at least 3 subtypes. The virus evolves rapidly and reports of high pathogenic variants of both Type 1 and Type 2 appearing in Europe, North America, and Asia have been reported within recent years. This abrupt occurrence of highly pathogenic PRRSV strains emphasizes the significance of monitoring the diversity of circulating strains around the world both in respect to the sensitivity and specificity of diagnostic tests as well as efficacy of available vaccines.

The aims and objectives of the PhD project will be introduced in the objectives section together with a short introduction to the situation of PRRSV in Denmark at the start of this study. The background chapter will provide a review on PRRSV with the emphasis on genetic diversity.

The results of the work performed during the PhD are presented in the four manuscripts included in the PhD thesis and a short summary of each manuscript is depicted below:

Manuscript I is focusing on the development of methods for complete genome sequencing of PRRSV. The sequencing strategy was based on the production of long range PCR fragments covering the PRRSV genome in two or four fragments with full-length cDNA as template. The sequencing of the PCR fragments was performed using Next Generation Sequencing (NGS) technologies and three different platforms were used. A total of 18 complete PRRSV genomes were obtained using this new method.

Manuscript II is focusing on the diversity of Type 1 PRRSV in Denmark. For the first time genetic and antigenic examinations of complete genomes of Danish isolated Type 1 PRRSV was conducted. Furthermore, extensive studies of ORF5 and ORF7 sequences were performed from 44 viruses collected from 2003 to February 2013. The diversity study confirmed that only Type 1 subtype 1 PRRSV is circulating in the Danish pig population. The examination of the Danish PRRS field viruses confirmed that there is a high overall diversity among Type 1 viruses in Europe. The phylogenetic study also indicated the presence of two Danish virus clusters, one dominating vaccine/LV like and one resembling an early introduced strain.

Manuscript III is focusing on the diversity of Type 2 PRRSV in Denmark. For the first time examinations of complete genomes of European isolated Type 2 PRRSV were performed. Furthermore, ORF5 and ORF7 sequences obtained from 57 viruses collected in the years 2003-2012 were examined. The diversity study confirmed that Danish Type 2 PRRS viruses share high genetic similarity to the vaccine strain and there was no obviously reason to believe that new Type 2 PRRSV strains have been introduced. However, a few viruses showed both a higher diversity to the other Danish viruses and to the vaccine strain and one virus harbored the largest deletion in NSP2 reported in Danish Type 2 PRRSV.

Manuscript IV is focusing on an experimental infection study in pigs with a Type 2 PRRS virus causing significant clinical disease in the field. Genetic and antigenic examination of ORF5 and partial NSP2 sequences obtained from the case virus revealed several variations compared to the vaccine strain. However, complete genome comparison of the case virus to the vaccine strain showed high genetic similarity and no obvious virulence maker was found. The results of the experimental infection study revealed that the strain induced only sparse clinical symptoms and the magnitude and duration of viraemia was comparable to an older Danish Type 2 strain. The results emphasized that infections in the field is often more severe than in experimental studies due to the multifactorial nature of PRRSV. Furthermore, the study underlined the need for more research on virulence markers of PRRSV.

DANSK SAMMENDRAG

I denne Ph.d.-afhandling præsenteres diversiteten af Porcine Reproductive and Respiratory Syndrom Virus (PRRSV) som cirkulerer i de danske grise. PRRS er en sygdom hos grise som er forårsaget af PRRS virussen som medfører forplantnings problemer hos søer og gylte og luftvejs problemer hos grise. På grund af genetisk heterogenitet, er PRRSV inddelt i to genotyper, Type 1 og Type 2. Type 1 PRRS virus er endvidere inddelt i mindst 3 undertyper. Virussen ændre sig hurtigt og indenfor de seneste år er der rapporteret om høj-patogene varianter af både Type 1 og Type 2 in Europa, Nord Amerika og Asien. Denne pludselige opståen af høj-patogene PRRSV stammer understreger, hvor vigtigt monitoreringen af diversiteten af de PRRSV stammer som cirkulerer rundt om i verdenen, med henblik på sensitiviteten og specificiteten af de diagnostiske analyser og ikke mindst effekten af de tilgængelige vacciner.

Formålet med dette Ph.d. projekt og en kort introduktion til situationen angående PRRSV i Danmark ved starten af projektet, vil blive omtalt i kapitlet 'Objectives'. Baggrundskapitlet vil give et overblik over PRRSV med vægt på den genetiske diversitet.

Resultaterne opnået under Ph.d. projektet er præsenteret i de 4 manuskripter som er inkluderet i denne Ph.d.-afhandling hvor et kort resumé er givet herunder:

Manuskript I fokuserer på udviklingen af metoder til brug for komplet genom sekventering af PRRSV. Sekventeringsstrategien var baseret på produktionen af lange PCR fragmenter som dækkede PRRSV genomet i to eller fire fragmenter hvortil fuld-længde cDNA blev brugt som template. Sekventeringen af PCR fragmenterne blev udført ved brug af 'Next Generation Sequencing' (NGS) teknologier hvor tre forskellige platforme blev brugt. I alt blev 18 komplette genomer sekventeret ved brug af denne nye metode.

Manuskript II fokuserer på diversiteten af Type 1 PRRSV i Danmark. For første gang blev komplette genomer af danske Type 1 PRRSV undersøgt på både genetisk og antigen niveau. Derudover blev omfattende studier af ORF5 og ORF7 diversiteten udført fra 44 virusser indsamlet fra 2003 til februar 2013. Fra dette studie blev det bekræftet, at det kun er Type 1 undertype 1 PRRSV som cirkulerer blandt de danske grise. Den videre undersøgelse af de danske PRRS virus bekræftede, at der er en overordnet høj diversitet blandt Type 1 virusser i Europa. De fylogenetiske studier viste tilstedeværelsen af to danske virus klynger, en dominerende vaccine/LV-lignende klynge og en klynge med virus som lignede en tidligere introduceret Type 1 PRRSV stamme.

Manuskript III fokuserer på diversiteten af Type 2 PRRSV i Danmark. For første gang blev komplette Type 2 PRRSV genomer isoleret i Europa undersøgt. Derudover blev ORF5 og ORF7 sekvenser fra 57 virusser indsamlet i årene 2003-2012 analyseret. Diversitetsstudiet bekræftede at de Danske Type 2 PRRS virusser deler en høj genetisk lighed til vaccine stammen og at der ikke var nogen åbenlys grund til at tro, at der er blevet introduceret nye Type 2 stammer til Danmark. Dog viste det sig at et par virusser havde en større diversitet både til de andre danske virusser og til vaccine stammen samt en virus der havde den længste rapporteret deletion i NSP2.

Manuskript IV fokuserer på et eksperimentelt infektionsforsøg i grise ved brug af en Type 2 PRRS virus som forårsagede alvorlig klinisk sygdom i besætningen. Genetiske og antigenetiske undersøgelser af ORF5 sekvenser og partiel NSP2 sekvenser sekventeret fra case virusser viste at disse virus havde flere variationer ved sammenligning med vaccine stammen. Sammenligningen af komplette genomer opnået fra casen mod vaccine stammen viste dog overordnet høj genetisk lighed mellem disse og ingen åbenlyse virulensmarkører blev fundet. Resultaterne fra det eksperimentelle infektionsforsøg viste, at case virussen kun kunne inducerer milde kliniske symptomer og styrken samt varigheden af viræmien var sammenlignelig med en ældre dansk Type 2 stamme. Resultaterne opnået her understreger at infektioner i felten ofte er mere alvorlige end set ved eksperimentelle infektioner grundet den multifaktorielle natur som PRRSV besidder. Yderligere så understreger dette studie behovet for yderligere forskning i virulensmarkører for PRRSV.

OBJECTIVES

The surveillance of the genetic and antigenic diversity of circulating PRRS viruses worldwide is very important for the advancement of vaccines and development and maintenance of diagnostic tools. Since 2010, the virology group at the National Veterinary Institute, DTU, Denmark has participated in an EU project: New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia (PoRRSCon). The final goal of the EU project is to develop new generation, efficacious and safe maker vaccines that can be adapted to temporary changes and geographical differences. Our contribution to the EU project lies in work package 3: Geno- and serotyping of PRRSV isolates/strains with Professor Lars E. Larsen as work package leader.

After the first incidence of PRRS in Denmark in 1992 and until 2002 all research and diagnostics of PRRSV at the National Veterinary Institute took place at the Virus department at the island Lindholm. In 2003 the PRRSV activities were moved to the department at Frederiksberg. Virus detection was at first carried out by inoculation of cell cultures followed by immunohistochemically staining but in the fall of 2007 implementation of PCR for PRRS virus detection was conducted.

Since the move of PRRSV activities to the department at Frederiksberg, the research on PRRSV has been minimal and there has not been published any ORF5 and ORF7 Type 1 or Type 2 PRRSV sequences obtained from viruses isolated since the late 1990s.

Regarding complete genome sequences, none Danish Type 1 or Type 2 PRRSV sequencing have been performed prior the start of this project. On European level, only 10 complete (or near complete) genomes of Type 1 PRRSV are as of this date public available and globally an additional eight sequences of Type 1 PRRSV genomes has been published. Of European isolated Type 2 PRRSV none complete genomes have to date, been made public available or even sequenced.

The export of Danish pigs to Eastern and central Europe is significant i.e. in 2012 Denmark exported more than 9 million living pigs and more than ¼ of a million breeding animals whereas less than 100 breeding animals were imported to Denmark. Despite this limited import of living pigs the possibility that foreign PRRSV isolates may be introduced into Denmark by contaminated transport carriers or persons cannot be excluded but this will probably be rare events. Nevertheless,

the diversity of PRRSV circulating in Denmark is of mutual interest for a range of European pig producing countries.

The main objective of this PhD was to develop methods and protocols for full genome sequencing of PRRSV in order to close the gap in knowledge on the genetic diversity of circulating Type 1 and Type 2 PRRS viruses in Danish pigs and to participate in the elucidation of the genetic variability of PRRSV genomes in Europe.

A more detailed knowledge of the variability of circulating Danish PRRSV ioslates are necessary for the evaluation of the sensitivity of diagnostic tests and for the evaluation of existing and future control of this important porcine pathogen.

1. BACKGROUND

1.1. The discovery of Porcine Reproductive and Respiratory Syndrome Virus

A mystery swine disease causing reproductive failure and respiratory disease was first described in the late 1980s in North America (Collins, 1991, Keffaber, 1989). A few years later a syndrome with similar clinical signs was observed in Western Europe (Terpstra et al., 1991, Wensvoort et al., 1991). During the winter of 1990-91 the disease appeared in Germany and in the Netherlands and since then spread through the rest of Western Europe (Wensvoort et al., 1991). In 1991, a large-scale laboratory investigation was conducted to search for the etiological agent and it was found that the disease causing agent was viral (Wensvoort et al., 1991). The first isolate of this agent was named Lelystad Virus (LV) after the Dutch city where it was isolated (Wensvoort et al., 1991). Shortly after the isolation of LV, a virus showing resembling clinical field signs was isolated in USA and designated American Type Culture Collection VR-2332 (ATCC VR-2332), hereafter referred to as VR-2332 (Collins et al., 1992).

Antigenic studies of LV and VR-2332 revealed antigenic differences between the European and North American isolated viruses, but also serological variations among the North American virus isolates were detected (Wensvoort et al., 1992). Genomic sequence analysis revealed significant genetic differences between viruses isolated in Europe and North America, with only 60 % nucleotide identity (Allende et al., 1999). These genomic differences confirmed the presence of two distinct genotypes, today known as Type 1 for the European isolates and Type 2 for the North American isolates.

Before the etiological agent causing the disease was known, the syndrome was given various names such as disease 89, pig plague 89, Swine Reproductive and Respiratory Syndrome (SRRS), Swine Infertility and Respiratory Syndrome (SIRS), Porcine Epidemic Abortion and Respiratory Syndrome (PEARS), Blue ear disease, Porcine Reproductive and Respiratory Syndrome (PRRS) (Goyal, 1993). However, at the First International Symposium on SIRS/PRRS held at St. Paul, Minnesota, USA, in 1992, it was decided to name the syndrome Porcine Reproductive and Respiratory Syndrome (PRRS) and its virus Porcine Reproductive and Respiratory Syndrome Virus (PRRSV).

Today, PRRSV is endemic in all pork producing countries with both genotypes distributed worldwide (Shi et al., 2010a).

1.2. Taxonomy

PRRSV is a small enveloped RNA virus belonging to the *Arteriviridae* family *(Benfield et al., 1992, Conzelmann et al., 1993, Wensvoort et al., 1991)*. Other members of this family are Equine Arteritis Virus (EAV), Lactate Dehydrogenase Elevation Virus (LDV) of mice, and Simian Hemorrhagic Fever Virus (SHFV). Based on the similarities in the genomic orientation and replication mechanism, the *Arteriviridae* family together with the *Coronaviridae, Roniviridae,* and *Mesoniviridae* families is placed in the order *Nidovirales* (Cavanagh, 1997).

1.3. Genomic organization

The genome of PRRSV is a single-stranded RNA molecule with a positive-sense orientation. The genome is 15-15.5 kb long with a 5'-end methylated cap structure and a 3'-end polyadenylated tail (Fig. 1). Untranslated regions (UTR) are present at both termini (Allende et al., 1999, Meulenberg et al., 1993, Meulenberg et al., 1998a). The genome encodes at least 10 open reading frames (ORFs), including the recently discovered ORF5a (Firth et al., 2011, Johnson et al., 2011, Meulenberg et al., 1993, Wu et al., 2001). ORF1a and ORF1b constitute about 75 % of the genome, and encodes two long non-structural polyproteins, pp1a and pp1ab, with the synthesis of the latter depending on a ribosomal frameshift near the 3'-end of ORF1a (Meulenberg et al., 1993, Snijder, 1998a). ORF2-5 encodes the membrane glycoproteins (GP), GP2-GP5, and ORF6 and ORF7 encodes a non-glycosylated membrane protein (M) and the nucleocapsid (N) protein, respectively. Two small genes, ORF2b and ORF5a, are fully embedded in ORF2 and depending on the genotype partially or fully embedded in ORF5, encodes the non-glycosylated proteins E and ORF5a protein, respectively (Firth et al., 2011, Johnson et al., 2011, Wu et al., 2001) (Fig. 1.1).

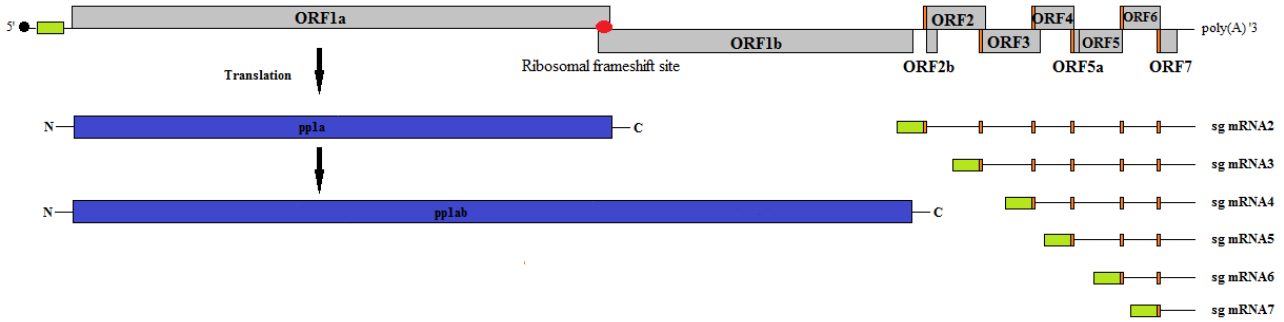

Fig. 1.1. Schematic representation of PRRSV genome orientation. *Each ORF encoded by the PRRSV genome is represented as a rectangle marked with the respective name of the gene. The 5' methylated cap structure is shown as a black sphere and the ribosomal frameshift is marked with a red sphere. The black lines at both termini represent the UTRs. The green box at the 5'-UTR represents the common leader sequence and the orange boxes located 5' to the ORF of the structural proteins represent the mRNA bodies. sg mRNA2-7 is shown to the right of the figure and the polyproteins pp1a and pp1ab are shown to the left.*

1.4. Structure

The PRRS virus has pleomorphic morphology. The virion has a spherical to oval shape with a size ranging from about 50 to 65 nm, a hollow, layered core of around 40 nm diameter and a smooth outer surface with the envelope protein complexes embedded (Spilman et al., 2009).

A schematic representation of the PRRS virion is illustrated in fig. 1.2. The genome is enclosed by the nucleocapsid which is constituted of a double-layered chain of nucleocapsid protein homodimers that bundle into a hollow ball (Spilman et al., 2009). The nucleocapsid core is surrounded by a lipid membrane, the envelope where the structural proteins are embedded (Benfield et al., 1992, Wensvoort et al., 1991). The major protein components of the lipid envelope are GP5 and M, which together encompass at least half the amount of the viral proteins. GP5 and M forms a disulfide-linked heterodimer through conserved cysteine residues in both proteins (Verheije et al., 2002). The minor structural proteins GP2, GP3, and GP4 forms a multimeric complex incorporated in the lipid envelope and for at least the Type 1 PRRS viruses E is also a part of this complex (Das et al., 2010, Wissink et al., 2005). The recently discovered ORF5a protein is believed to be the eighth structural protein of PRRSV, but its orientation in the virion particle and its interaction with the other structural proteins still needs to be clarified (Firth et al., 2011, Johnson et al., 2011).

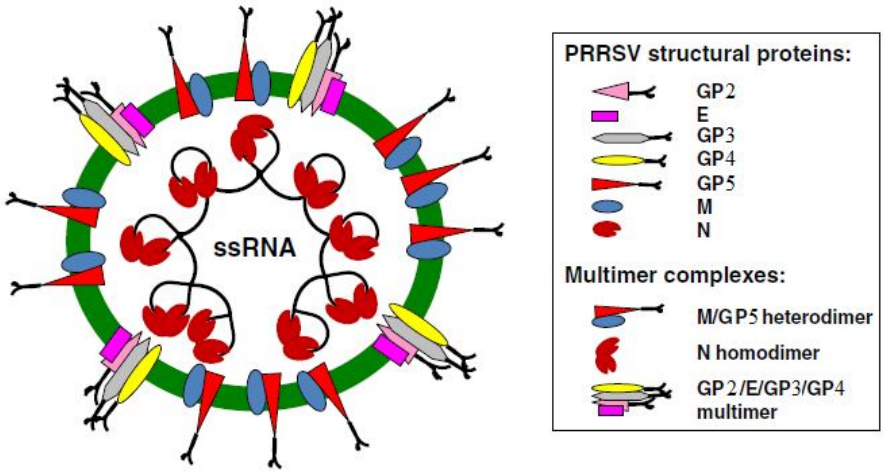

Fig. 1.2. Schematic representation of the PRRSV virion. *The orientation of the structural proteins of GP2, E, GP3, GP4, GP5, M, and N protein are shown. GP5/M forms a heterodimer and the minor glycoproteins and E forms a multimeric complex. The N protein homodimers are shown surrounding the PRRSV RNA genome. The figure is modified from Music and Gagnon (2010).*

1.5. Stability

Because PRRSV posses a lipid envelope, the survivability outside the host is affected by temperature, pH, and detergents. PRRSV is heat labile, but relatively stable at 4°C and -70°C and solvents such as chloroform and ether are particularly efficient at disrupting the lipid envelope (Benfield et al., 1992). pH values between 6.5-7.5 keeps the virus stable however below and above this pH range the virus' infectivity is reduced (Bloemraad et al., 1994).

1.6. Attachment and entry into host cells

PRRSV has a very restricted cell tropism *in vivo* and *in vitro*. *In vivo,* PRRSV only replicate within macrophages in the respiratory and lymphoid systems of the pig (Halbur et al., 1995a, Halbur et al., 1995b, Duan et al., 1997).

PRRSV entrance into the host cell occurs by receptor-mediated endocytosis (Duan et al., 1998). Initial binding of PRRSV to its host cell occurs via interactions with heparan sulphate glycosaminoglycans (GAGs) (Delputte et al., 2005). This interaction with heparan sulphate GAGs does not internalize the virus particles, but concentrate the virions at the cell surface (Delputte et al., 2005). The receptor involved in internalization of PRRSV, is the sialoadhesin (Sn) receptor (Vanderheijden et al., 2003). The binding of the virus particle to the Sn receptor occur through

binding of sialic acid present on the viral M/GP5 complex (Van Breedam et al., 2010b). Upon binding of the virus to the Sn receptor, the virus is internalized via clathrin-mediated endocytosis and hence enters the endocytic pathway (Nauwynck et al., 1999, Vanderheijden et al., 2003). After entry into the host cell, PRRSV enters the early endosome where it co-localizes with the scavenger receptor CD163 (Van Gorp et al., 2009). The receptor CD163 together with at drop in pH is believed to be involved in the uncoating of the virus and release of its genome into the cytosol (Van Gorp et al., 2008, Van Gorp et al., 2009). The exact mechanism for this uncoating and the role of CD163 is not yet clear. However, it has been shown that two of the minor PRRSV glycoproteins embedded in the virion surface, GP2 and GP4, interact with the CD163 receptor (Das et al., 2010).

In vitro, PRRSV are able to proliferate in the primary cell lines porcine alveolar macrophages (PAM) and blood monocytes (Voicu et al., 1994, Wensvoort et al., 1991). Two non-porcine cell sub-clones; Marc-145 and CL2621 cells, both derived from the African green monkey cell line MA104, are routinely used for *in vitro* propagation of PRRSV (Bautista et al., 1993, Kim et al., 1993). However the infection process in these cell lines differs from porcine macrophages since the MA104 cell line does not express the Sn receptor and the virus propagation in this cell line can lead to new mutations in the structural proteins to improve infection (Van Breedam et al., 2010a, Nauwynck et al., 2012).

1.7. Replication and translation

The replication of the PRRSV genome takes place in the cytoplasm of the host cell. The proteins involved in replication are encoded in the two major ORFs, ORF1a and ORF1b. Before the replication of the viral genome can take place, the viral proteins involved in this process have to be synthesized. ORF1a is translated directly from the genomic RNA to the polyprotein 1a (pp1a) where ORF1b is translated through a -1 ribosomal frameshift just upstream of the ORF1a termination codon resulting in the synthesis of polyprotein 1ab (pp1ab) from ORF1ab (Snijder and Meulenberg, 1998). After the protein synthesis, the polyproteins are cleaved into 14 functional nonstructural proteins (nsps). The key enzymes for the RNA replication are the RNA-dependent RNA polymerase (RdRp) and the RNA helicase both encoded in ORF1b (nsp9 and nsp10) (van Dinten et al., 1996). The structural proteins are not translated from the genomic RNA, as the 3' proximal third of the genome is not accessible for ribosomes involved in genome translation, but instead the structural proteins are translated from a nested set of subgenomic (sg) mRNA's (sg mRNA2-7) (Pasternak et al., 2006, Conzelmann et al., 1993) (Fig. 1.1). The replication of the

genomic RNA (mRNA1) starts with the synthesis of full-length minus-strand RNA by the binding of the RdRp enzyme complex to RNA signals at the genomic 3' end. The full-length minus-strand RNA molecule then acts as template for the synthesis of positive-sense RNA, the genomic RNA (Snijder and Meulenberg, 1998). The sg RNA templates for sg mRNA synthesis are believed to be generated by discontinuous minus-strand RNA synthesis. The 5'terminal common leader sequence derived from the 5'-UTR region of mRNA1 is fused with mRNA bodies located at the 5' end of the structural ORFs (Pasternak et al., 2006) (fig. 1.1). Each sg mRNA2-6 is structurally polycistronic (except sg mRNA7 only encodes ORF7) but only the 5' proximal ORF is translated into protein and hence functionally monocistronic (Pasternak et al., 2006). PRRSV RNA synthesis is asymmetric and produces more positive-sense RNA than minus-sense RNA (Sawicki et al., 2001).

1.8. Non-structural proteins, - processing and function

The ORF1a and ORF1ab encode the two long non-structural proteins, pp1a and pp1ab (Snijder, 1998b). The pp1a and pp1ab are processed into 14 non-structural proteins (nsp1α, nap1β, nsp2-6, nsp7α, nsp7β, and nsp8-nsp12) by a complex proteolytic cascade that is directed by four proteinase domains encoded in ORF1a (Fang and Snijder, 2010, Ziebuhr et al., 2000). The four proteases involved in the cleavage of the polyproteins are nsp1α, nap1β, nsp2, and nsp4 (Ziebuhr et al., 2000). The first step in the processing of pp1a or pp1ab is the rapid auto-release of nsp1α nsp1β. These proteases both contain a papain-like cysteine protease domain, called PCPα and PCPβ respectively, and cleave themselves from the polyprotein at their C-terminal junctions (den Boon et al., 1995, Chen et al., 2010). The nsp2/nsp3 proteolytic cleavage is processed by a cysteine protease located at the N-terminal domain of nsp2 (den Boon et al., 1995). After release of nsp1α, nsp1β, and nsp2, the remaining cleavages in both pp1a and pp1ab are carried out by the viral 'main' protease, the nsp4, a 3C-like protease (Snijder et al., 1996).

Even though the non-structural proteins constitute more than ¾ of the PRRSV genome, little is known about their function (Chen et al., 2010). The nsp1α is believed to be important for sg mRNA synthesis where as nsp1β is mostly required for genome replication (Kroese et al., 2008). Furthermore, nsp1α and nsp1β have been found to be involved in the blockage of the type I interferon synthesis and signaling pathway (Chen et al., 2010). nsp2 is the largest of the non-structural proteins although it vary in length as both deletions and insertions are common in this region of the PRRSV genome (Fang et al., 2004, Han et al., 2006, Gauger et al., 2012). The amino acid variation of the nsp2 protein is also very high both within the same genotype and between the

two genotypes (Fang et al., 2004, Han et al., 2006, Gauger et al., 2012, Allende et al., 1999). Besides being involved in the proteolysis of pp1a and pp1ab nsp2 may also be a potential interferon (IFN) antagonist and involved in replication-associated membrane rearrangements in collaboration with nsp3 (Fang and Snijder, 2010). nsp2 is the viral protein containing the highest frequency in B-cell epitopes (Oleksiewicz et al., 2001, de Lima et al., 2006, Yan et al., 2007) and the humoral antibody response to nsp2 is greater than towards any other PRRSV protein, however it is non-neutralizing (Han et al., 2007, Johnson et al., 2007, Brown et al., 2009).

The pp1a protein products nsp5-8 are not well described and have currently unknown function and structure (Dokland, 2010). Nevertheless a recent study showed that at 6 hours PI most of the nsps co-localized in the cell cytoplasm, at a perinuclear site assumed to be the site of the replication complex (Chen et al., 2010, Snijder and Meulenberg, 1998). ORF1b encodes the key enzymes for RNA replication. The nsp9 encodes the RNA-dependent RNA polymerase (RdRp) and nsp10 encodes the helicase involved in the unwinding of double-stranded RNA (dsRNA) (van Dinten et al., 1996). The nsp11 encodes a nidovirus endoribonuclease (NendoU) domain and have a critical role in the replicative cycle (Posthuma et al., 2006). Nsp12 is of unknown structure and function (Fang and Snijder, 2010).

1.9. The structural proteins

PRRSV encodes at least seven structural proteins: GP2, E, GP3, GP4, GP5, M, and N protein (fig. 1.2). An eight structural protein, ORF5a protein, was recently discovered, however its function and location among the structural proteins is unknown (Firth et al., 2011, Johnson et al., 2011).

GP2 is encoded in ORF2, constitute 249 and 256 amino acids in Type 1 and Type 2 PRRSV respectively. The protein contains two highly conserved N-glycosylation motifs (N-X-S/T-X, X ≠ P) at N173 and N179 for Type 1 and N178 and N184 for Type 2 (Dea et al., 2000). The importance of the N-glycosylation of GP2 has been studied. For the Type 1 PRRSV, the role of N-glycosylation of GP2 showed that lack of glycosylation at either one or both sites did not affect the formation or infectivity of virions (Wissink et al., 2004). On the other hand for Type 2, the N-glycosylation at N184 was required for infectious virus production (Das et al., 2011). GP2 has been found to interact with the CD163 receptor, thus GP2 may be involved in the uncoating and genome release of PRRSV (Das et al., 2010).

The E protein is synthesized from the same sg mRNA (mRNA2) as GP2 and is 70 and 73 amino acid long for Type 1 and Type 2 PRRSV, respectively (Wu et al., 2001). The protein is

unglycosylated and has a central hydrophobic domain and a hydrophilic C-terminus (Snijder et al., 1999). The E protein likely functions as an ion-channel protein and allows ions to enter the virion and hereby triggering internal capsid disassembly and promote the release of the viral genome into the cytosol (Lee and Yoo, 2006). The expression of GP2 and E as enhanced green fluorescent fusion proteins has shown that E is preferentially expressed which suggest that E is the principal product of ORF2 (Wu et al., 2001).

GP3 is encoded by ORF3 and the mature protein is 265 and 254 amino acid long in Type 1 and Type 2, respectively. GP3 is the most variable structural protein and Type 1 GP3 is prone for deletion in a highly variable region located at the C-terminal (Oleksiewicz et al., 2000, Darwich et al., 2011). The GP3 is heavily N-glycosylated and contains seven N-glycosylation motifs (N27, N42, N50, N130, N151, N159, and N 194 for Type 1 and N29, N42, N50, N131, N152, N160, N196 for Type 2) (Dea et al., 2000). Recent findings revealed that the first six N-glycosylation sites in GP3 are glycosylated in Type 2 PRRSV (Das et al., 2011). The seventh N-glycosylation site, N195, did not constitute glycosylation however this position is believed to be located in the transmembrane region of GP3, hence unlikely to be glycosylated (Das et al., 2011). Glycosylation at N42, N50, and N131 for Type 2 PRRSV is necessary for infectious virus production (Das et al., 2011). GP3 of Type 1 PRRSV encodes a B-cell epitope at aa60-87 which has been shown to induce neutralizing antibodies (Oleksiewicz et al., 2001, Oleksiewicz et al., 2002). For Type 2 PRRSV, four overlapping B-cell epitopes has been predicted at position aa61-105 and were strongly immunoreactive to tested sera (de Lima et al., 2006).

GP4 is encoded by ORF4 and is 183 and 178 amino acid long for Type 1 and Type 2 PRRSV, respectively. The 5’ end of ORF4 and the 3’ end of ORF3 coding regions overlap and hence any deletion found in ORF3 will also affect ORF4. The GP4 protein encodes four N-glycosylation motifs, N37, N 88, N124, and N134 for Type 1 PRRSV and N37, N84, N120, and N130 for Type 2 PRRSV (Dea et al., 2000). Any three of the four putative N-glycosylation sites (N37, N84, N120, and N130 in GP4 PRRSV Type 2) has been found to be necessary for recovery of infectious virus (Das et al., 2011). Together with GP2, GP4 has been found to interact with the CD163 receptor, thus GP4 may be involved in the uncoating and genome release of PRRSV (Das et al., 2010). GP4 of Type 1 PRRSV encodes a B-cell epitope at position aa58-70 that induces neutralizing antibodies (Oleksiewicz et al., 2001, Oleksiewicz et al., 2002). For Type 2, a B-cell epitope has been predicted however the ability to induce neutralizing antibodies needs more research (de Lima et al., 2006).

The minor structural proteins GP2, GP3, and GP4 are all required for generation of infectious virions (Wissink et al., 2005). The three minor glycoproteins forms a multimeric complex incorporated in the lipid envelope and for at least the Type 1 PRRS viruses E is also a part of this complex (Das et al., 2010, Wissink et al., 2005). The recently discovered ORF5a protein is believed to be the eighth structural protein of PRRSV, but its orientation in the virion particle and its interaction with the other structural proteins still needs to be clarified (Firth et al., 2011, Johnson et al., 2011).

GP5 is the most abundant glycoprotein found on the surface of the PRRSV virion and form a heterodimer with M protein (Van Breedam et al., 2010b). GP5 is encoded by ORF5 which is one of the most variable regions of the PRRSV genome and it is the most examined gene in regard to diversity studies (Shi et al., 2010a, Shi et al., 2010b). The GP5 harbors various numbers of putative N-glycosylation sites where glycosylation at N46 and N53 for Type 1 PRRSV and at N44 and N51 for Type 2 PRRSV are highly conserved (Music and Gagnon, 2010) (Manuscript II and III). N-glycosylation at position N44 in Type 2 PRRSV has previously been shown to be important for infectious virus production (Ansari et al., 2006). The GP5 harbors a neutralizing epitope at position aa38-54 for Type 1 PRRSV and at position aa37-47 in Type 2 PRRSV (Oleksiewicz et al., 2002, Ostrowski et al., 2002, Plagemann, 2004a, Plagemann, 2004b). For Type 2 PRRSV strains the GP5 also contains a decoy epitope at position aa27-30 which is not neutralizing but may function to distract the humoral immune response hence delaying the induction of neutralizing antibodies against PRRSV (Ostrowski et al., 2002).

The M protein encoded by ORF6 is composed of 173 and 174 amino acid residues in Type 1 and Type 2 PRRSV, respectively. The M protein is unglycosylated and is highly conserved among the structural proteins (Meulenberg et al., 1993, Mardassi et al., 1995) (Manuscript II and III). The M protein and GP5 forms disulfide-linked heterodimers through conserved cysteine residues in both proteins. This conformation of M and GP5 is essential for virus infectivity (Van Breedam et al., 2010b).

The N protein encoded by ORF7 is the most abundant viral protein in the infected cell (Dea et al., 2000, Snijder and Meulenberg, 1998). The N protein has shown size polymorphism for the Type 1 PRRSV and previous reports have suggested that the length of ORF7 could be a subtype marker (Stadejek et al., 2008, Jackova et al., 2012). The N protein is highly immunogenic in pigs although not neutralizing (Diaz et al., 2009, Meulenberg et al., 1998b).

1.10. Pathogenesis

By definition, PRRSV is not a 'persistent' virus. Nevertheless, since the average lifetime of production pigs is 180 days, PRRSV infection is 'life-long' for the majority of pigs (Chand et al., 2012). Within a production system, PRRSV infection predominantly exists as a subclinical infection, participating as a co-factor in various polymicrobial disease syndromes, such as Porcine Respiratory Disease Complex (PRDC) and Porcine Circovirus Associated Disease (PCVAD) (Chand et al., 2012).

PRRSV has been recovered from a variety of porcine secretions and excretions including blood, semen, saliva, feces, aerosols, milk, and colostrum (Rossow et al., 1994, Swenson et al., 1994, Wagstrom et al., 2001, Wills et al., 1997b). Fecal shedding remains a highly debated issue as studies reports the presence of PRRSV in feces from 28 to 35 days post infection (PI), whereas others report no detection of virus in fecal samples (Wills et al., 1997b, Yoon et al., 1993).

Transmission of PRRSV primarily occurs through direct contact between the infected and naïve pigs (Rossow, 1998). Aerosol transmission is also a route of transmission of PRRSV (Wills et al., 1997a, Kristensen et al., 2004), although experimental studies have shown that effective aerosol transmission may be influenced by the pathogenicity of the circulating viruses (Cho et al., 2007). Transmission of PRRSV to sows and gilts via semen from infected boras is also a contagious risk (Yaeger et al., 1993, Christopher-Hennings et al., 1995).

In the acute PI phase, PRRS virus replicate in alveolar and other tissue macrophages (Pol et al., 1991). The acute PI phase is characterized by high viral load in tissue and by the presence of viremia in serum, which may last up to 28 days PI (Halbur et al., 1995a, Halbur et al., 1995b, Duan et al., 1997). Past the acute PI stage, a persistent phase of infection follows with lymphoid tissues as the primary site of virus replication. PRRS virus can be isolated from lymph nodes for more than 100 days PI and virus is easily transmitted to naïve pigs during asymptomatic periods (Horter et al., 2002, Rowland et al., 2003).

1.11. Clinical signs and pathology

The clinical presentation and clinical signs of PRRS varies greatly between herds. Infection with PRRSV shows two different sets of clinical signs: reproductive and respiratory (Keffaber, 1989, Wensvoort et al., 1991). The clinical presentation of PRRSV infections depends on the age of the pig infected and on the pregnancy status and trimester of gestation of the infected sow/gilt (Rossow,

1998). Studies found that pigs experimentally infected with nine different isolates of Type 2 PRRSV had major differences in clinical disease, rectal temperatures, and gross and histological lung lesions (Halbur et al., 1995a, Halbur et al., 1995b, Halbur et al., 1996). Animals infected with mildly virulent isolates or the LV had transient pyrexia, dyspnea and tachypnea, whereas infection with highly virulent isolates induced labored breathing, pyrexia, lethargy, and anorexia. Furthermore, studies have reported that the impact on reproductive performance may be isolate dependent (Halbur et al., 1995a, Halbur et al., 1995b, Mengeling et al., 1996).

The clinical signs in sows are characterized by inappetence, anorexia, and reproductive disorders such as abortion, premature birth, birth of dead or weak piglets, and foetal death with or without mummification. A less frequently observed sign is transient blue discoloration of, the ears, abdomen, or vulva (Terpstra et al., 1991).

PRRSV infection in weaned pigs is characterized by fever, pneumonia, lethargy, and failure to thrive (Rossow, 1998).

Gross lesions observed following PRRSV infection vary widely and may be dependent on the virus isolate, genetics of the infected pig, and stress factors (environment and health status of the pig herd). Lung lesions vary from none to diffuse consolidation and are commonly complicated by lesions resulting from concurrent bacterial infections which can localize separate or intermixed (Rossow, 1998).

1.12. Immune response

The pig's adaptive immune response against PRRSV is characterized by being delayed and defective (Beura et al., 2010). Following a natural infection, it takes at least 3 months to reach immunity peak levels and it does not appear to be solid enough to prevent reinfection, especially if the reinfection is caused by antigenically heterologous PRRSV strains (Murtaugh et al., 2002, Zuckermann et al., 2007). Pigs infected with PRRSV fail to generate any significant inflammatory cytokine expression in the lungs, including the type I interferons (IFN-α/β), interleukin (IL)-1, and TNF-α (Thanawongnuwech et al., 2001, Van Reeth et al., 1999). The expression of type I interferon is important for the activation of innate immune response (Kimman et al., 2009). The downregulation of INF-α can be a crucial step in PRRSV pathogenesis as INF-α has been shown to inhibit PRRSV replication (Albina et al., 1998, Le Bon et al., 2001), hence the weak initial innate

immune response may lead to longer survival of the virus in the infected animal (Kimman et al., 2009).

Following infection, the earliest and strongest antibody response is directed against the N protein which is measureable 5-9 days PI (Johnson et al., 2004, Kimman et al., 2009). Antibodies against the two non-structural proteins nsp1 and nsp2 are evident at 14 days PI, and reach peak levels at 28-35 days PI (Johnson et al., 2007, Brown et al., 2009, de Lima et al., 2006, Oleksiewicz et al., 2001). All these early produced antibodies are non-neutralizing whereas the neutralizing antibodies first appear 4 weeks PI or even later (Lopez and Osorio, 2004). The neutralizing antibody response against the GP5 neutralizing epitope is weak and delayed, and some animals fail to make a detectable antibody response against GP5 (Chand et al., 2012, Yoon et al., 1994). The mechanism for the weak antibody response towards GP5 is linked to the N-glycosylations surrounding the neutralizing epitope, a phenomenon called N-glycan shielding (Chand et al., 2012). Furthermore, for Type 2 PRRSV, GP5 encodes a decoy epitope at position aa27-30 which is not neutralizing but may function to distract the humoral immune response hence delaying the induction of neutralizing antibodies against PRRSV (Ostrowski et al., 2002).

1.13. The origin of PRRSV

When and where PRRSV came from is still a mystery. It is assumed that PRRSV has evolved from LDV, it's most similar *Arteriviridae* relative, however PRRSV does not infect rodents and LDV has not been found in pigs (Murtaugh et al., 2010). Even though there were no reports of clinical outbreaks of PRRS before 1987 in North America and 1990 in Europe, antibodies against PRRSV has been detected in archive serum samples originated from East German herds in 1988 and as early as 1979 in Canadian herds (Carman et al., 1995).

Despite the concurrence in emergence and disease symptoms, viruses isolated from the two continents were genetically distinct and divided into two genotypes, today known as Type 1 for the European isolates and Type 2 for the North American isolates. There is no unanimous hypothesis that explains the simultaneous emergence of the virus however it is commonly assumed that they genetically diversified in separate reservoirs prior to emergence (Nelsen et al., 1999, Plagemann, 2003). Studies to find the most resent common ancestor estimated that Western Europe PRRSV isolates existed in 1980, approximately 10 years before the epidemic emergence (Forsberg et al., 2001, Forsberg, 2005). Furthermore, due to the genetic diversity between the Type 1 viruses, it was found that the Western Europe epidemic was caused by transfer of several lineages from the

original reservoir (Forsberg et al., 2001). The likely origin of transfer was located in Eastern Europe, as Eastern European pigs harbored highly diverse Type 1 PRRSV lineages, that was shown to diversify before the Western Europe Type 1 PRRSV lineages (Stadejek et al., 2002). Thus, a plausible hypothesis is that Type 1 PRRSV originated in Eastern European pigs and was transmitted to Western European pigs following the increased East-West European contact that began in the late 1980s (Stadejek et al., 2002). The division of PRRSV into two distinct genotypes was proposed to occur as recently as 1980 (Hanada et al., 2005) however this estimate was questioned by Forsberg (2005) who on the basis of ORF3 sequences estimated the most resent common ancestor around 1880 although caution should be taken for this estimate as well (Forsberg, 2005). A more plausible hypothesis was proposed that PRRSV originated in European wild boars and that the virus was transferred to North America through these animals when imported in 1912 (Plagemann, 2003). This hypothesis may explain the long period of independent evolution on the two continents and implies a divergence time in agreement with the estimations performed by Forsberg (2005).

1.14. Geographical distribution

PRRSV is endemic in the majority of the pork producing countries, and both genotypes are distributed worldwide (Shi et al., 2010a). A PRRSV Type 1 isolate was identified in a swine herd in the United States in 1999 (Fang et al., 2004, Ropp et al., 2004) and Type 1 PRRSV has been introduced to four other non-European countries beside U.S.A. These countries are Canada (Dewey et al., 2000), South Korea (Lee et al., 2010), China (Jiang et al., 2000), and Thailand (Thanawongnuwech et al., 2004).

PRRSV Type 2 was introduced in Europe in 1996 probably through the use of a live attenuated vaccine that reverted back to virulence in Danish pig herds (Botner et al., 1997, Madsen et al., 1998). Subsequently, PRRSV has been reported sporadically in the rest of Europe (Stadejek et al., 2013). Type 2 PRRS also circulate throughout most of Asia (An et al., 2007, Feng et al., 2008).

1.15. Genetic diversity

At first it was believed that Type 2 PRRS viruses were more genetically diverse, while Type 1 PRRS viruses exhibited a lower degree of variations (Meng et al., 1995, Kapur et al., 1996, Suarez et al., 1996). This perception changed following extensive sampling of Type 1 viruses which

revealed an even greater diversity among European isolated viruses than North American isolated viruses (Stadejek et al., 2002, Stadejek et al., 2006, Stadejek et al., 2008).

The first PRRSV isolated in Europe following the first epidemic was the Dutch isolate LV and this virus was then regarded as the protogenotype of Type 1 PRRSV (Wensvoort et al., 1991). However the genetic differences between the Type 1 viruses soon became evident and consequently LV could not be viewed as the ancestral virus type for all the Type 1 viruses isolated in Europe (Drew et al., 1997, Forsberg et al., 2002, Le Gall et al., 1998, Suarez et al., 1996, Wensvoort et al., 1991). Country-specific clusters were formed by viruses isolated in Great Britain, Italy, and Denmark (Forsberg et al., 2002, Frossard et al., 2013).

Further sampling and sequencing revealed even more diversity among the Type 1 PRRSV isolated in Europe. Especially, the viruses isolated in Eastern Europe showed high divergence and diversity compared to Western European isolates and a subtyping of Type 1 PRRS viruses were proposed by Stadejek et al. (2002, 2006, 2008). Originally, 3 subtypes were defined on the basis of their ORF5 sequences (Stadejek et al., 2006). However, ORF7, the ORF encoding the nucleocapsid have shown size polymorphism and hence it was recommended as a stable and independent indicator for subtyping and on the basis of the length of ORF7 it was possible to confirm the previous identified subtypes using this classification (Stadejek et al., 2008). Nevertheless, caution may be taken when subtyping based on the size polymorphism of ORF7 since a recently isolated virus from Slovakia encoded a 132 amino acid N protein (compared to 128 amino acids N protein in the LV) but otherwise resembled the subtype 1 PRRSV (Jackova et al., 2012).

Type 1 PRRSV has been introduced to 5 countries outside Europe and so far only subtype 1 has been detected (Shi et al., 2010a). Several Thai and Chinese viruses showed to be genetically related to two types of live-attenuated vaccine strains which was surprising as Type 1 live-attenuated vaccines was not officially applied in these countries (Thanawongnuwech et al., 2004).

The global evolutionary history of Type 1 PRRS viruses based on their ORF5 sequences was illustrated by Shi et al. (2010a) by phylogenetic analysis (fig. 1.3.). Here, the diversity among Type 1 subtype 1 viruses is evident, as it was possible to further divide this subtype into 12 clades (A-L, fig. 1.3.). The diversity of Type 1 PRRSV isolated outside Europe suggests multiple introductions of PRRSV in these countries, confirmed by the appearance of viruses from the same country in different clades (Shi et al., 2010a).

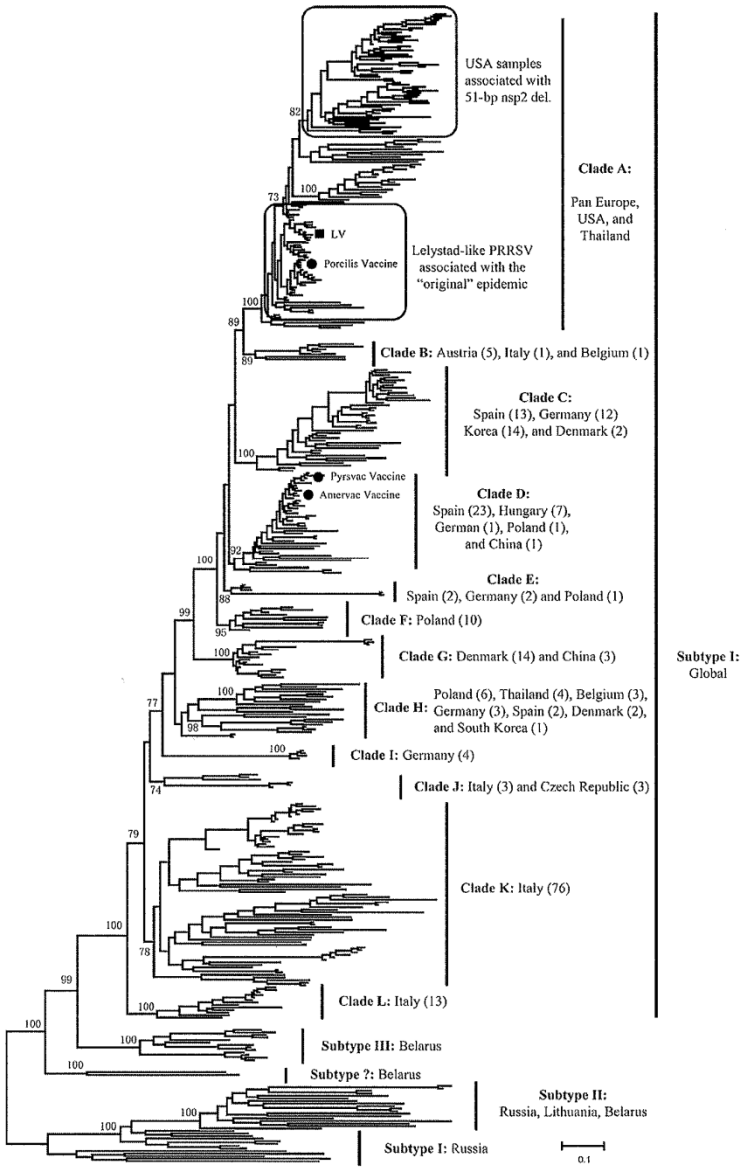

Fig. 1.3. Global phylogenetic analysis of Type 1 PRRSV ORF5 sequences. *For Western European subtype 1, a further division into clades (A-L) was possible and the Nationality of the viruses is stated on the right. The numbers in brackets corresponds to the number of sequences within the clade. The other Type 1 subtypes are orientated in the bottom of the tree. The figure is borrowed from Shi et al. 2010a.*

On the basis of ORF5 sequences, Type 2 PRRSV can be divided into 9 different lineages (fig. 1.4.) (Shi et al., 2010b). The Type 2 viruses isolated in North America showed the greatest diversity as they were distributed throughout seven of the nine lineages (fig. 1.4). Lineage 3 and 4 only included Asian isolated viruses. Within the seven lineages constituting the North American viruses,

Asian and European isolated viruses were also represented. Lineage 5 is the cluster with most countries represented, including Denmark. This lineage also harbored the Ingelvac MLV vaccine, which is the preferred vaccine against Type 2 PRRSV in most European countries (Stadejek et al., 2013) and the Type 2 protogenotype VR-2332 (Collins et al., 1992). The European interest for the diversity of Type 2 PRRSV isolated in Europe has been minor, since so far the majority of Type 2 PRRSV circulating in the European pig herds were vaccine-like (> 98 % identical) (Greiser-Wilke et al., 2010, Stadejek et al., 2013). However, in Hungary two Type 2 PRRSV isolates showed major divergence compared to the vaccine-strain on the basis of partial ORF5 sequences and the origin of these viruses could not be explained (Balka et al., 2008).

1.16. Quasispecies and recombination

The simplest explanation for the diversity of PRRSV is the RNA polymerase infidelity, as the RdRp does not harbor a proof-reading mechanism and mutation rates of the RdRp has been measured to roughly 10^{-4} (Lauring and Andino, 2010). This infidelity of the RdRp results in the formation of quasispecies (Lauring and Andino, 2010). Quasispecies of PRRSV frequently occur during replication (Rowland et al., 1999, Schommer and Kleiboeker, 2006, Zhao et al., 2012). However the survival of a quasispecies and evolution to a stable PRRSV strain depends on the mutations, as distribution of viral strains depends on how the viruses fits into the environment; popularly speaking: it is survival of the fittest (Lauring and Andino, 2010).

Another mechanism explaining the high diversity of PRRSV is recombination. Some viruses may belong to one clade/lineage on the basis of one gene and then to another clade/lineage when investigating another gene. This phenomenon was seen for Russian Type 1 viruses which were classified to belong to subtype 1, originally on the basis of their ORF7 length and phylogeny, however phylogenic analysis of their ORF5 sequence placed them further away from subtype 1 than subtype 2 and 3 (fig. 1.3) (Shi et al., 2010a). This diversity of the Russian viruses may be explained by recombination. Recombination plays an essential role for PRRSV biology since it is required for the formation of sg mRNA (Conzelmann et al., 1993, Pasternak et al., 2006). If a host cell is infected with two different PRRSV viruses at the same time, recombination between these two could occur during genomic replication and a scenario as with the Russian viruses could be the result. On the other hand, if the two viruses were genetically alike, the recombination genomes would be indistinguishable from non-recombinant genomes (van Vugt et al., 2001, Murtaugh et al., 2010). Recombination preferentially occurs between genomes with high similarity (van Vugt et al.,

2001). The Russian subtype 1 viruses may be a product of two ancestral variants in Russia, one closely related to the Western Europe subtype 1 viruses and one distantly related. The resultant recombinant then became prevalent in the field and the two parental viruses went extinct (Shi et al., 2010a).

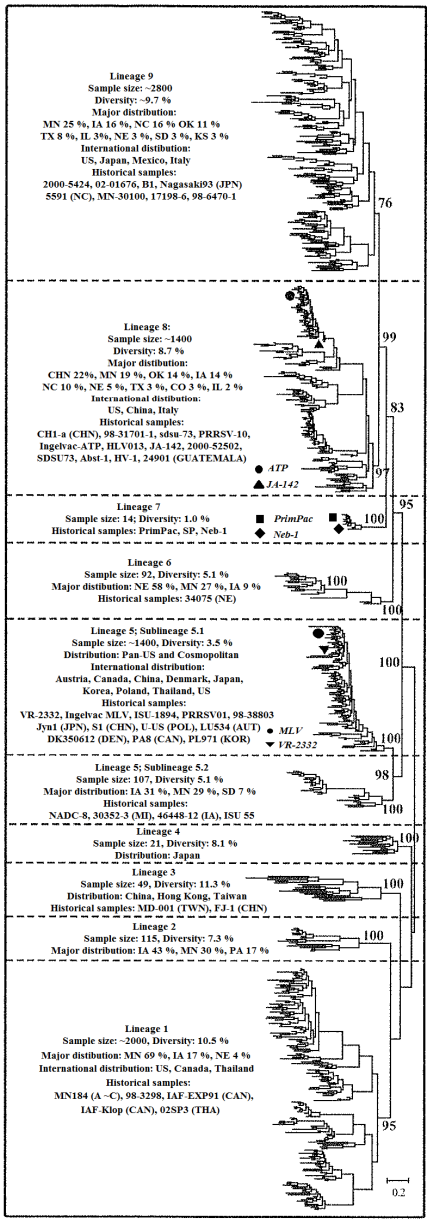

Fig. 1.4. Global phylogenetic analysis of PRRSV Type 2 ORF5 sequences. *Each of the nine lineages representing the diversity of Type 2 PRRSV ORF5 sequences are separated with a dotted line. One the left, sample size, diversity within the lineage, major distribution, and historical samples are listed. The figure is modified from Shi et al (2010b).*

1.17. High Fever Disease

From time to time highly virulent PRRSV strains evolve, which results in poor welfare for the pigs and huge economical losses for the swine industry (Han et al., 2006, Gauger et al., 2012, Karniychuk et al., 2010, Neumann et al., 2005).

In China in 2006, a severe 'high fever' disease occurred in several pig farms and subsequently overwhelmed almost half of China (Tian et al., 2007). The list of clinical symptoms was long: rubification, blood spots, petechiae, erythematous blanching rashes, and pimples, frequently observed in ears, mouth, noses, back, and the inner thigh. Other common symptoms were high fever (40-42°C), depression, anorexia, cough, asthma, lameness, shivering, and disorder in the respiratory tract, diarrhea, and subsequently death. Initially, the 'high fever' was suspected to be caused by hog cholera or African swine fever and PRRSV was not suspected as the etiology agent because many grown pigs died, which is unlike typical PRRSV infections (Tian et al., 2007). The course of the disease varied from 5 to 20 days, and pigs infected were highly contagious affecting the whole pig herd within 3-5 days. The epidemic persisted for several months partly due to the limited knowledge of the etiological agent (Tian et al., 2007). According to the China Animal Disease Control Center (CADC), the epidemic affected more than 2 million pigs with more than 400,000 fatal cases in just 4 month of duration (Tian et al., 2007). The etiological agent was found by extensive and systematic investigations, and by PCR and immunohistochemistry experiments the 'high fever' agent was determined to be PRRSV (Tian et al., 2007). Today the atypical PRRSV strain is referred to as highly pathogenic PRRSV (HP-PRRSV). Shortly after the epidemic of HP-PRRSV in China, it rapidly spread to pigs in Southeast Asia and nearby countries (An et al., 2011).

Phylogenetic analysis of HP-PRRSV ORF5 sequences revealed that they were very similar to typical Chinese PRRSV isolates (Tian et al., 2007). To study the evolutionary origin of the HP-PRRSV, complete HP-PRRSV genome sequences were compared to typical Chinese PRRSV genomes and it was suggested that the HP-PRRSV had gradually evolved from an early Chinese PRRSV, CH-1a, and the evolutionary path could be traced through intermediate PRRSV isolates (An et al., 2011, Feng et al., 2008). Furthermore, genetic analysis found in all HP-PRRSV isolates examined, a distinct 1 plus 29 amino acid deletion in nsp2 (Tian et al., 2007) which at first was believed to be a virulence marker, however chimeric infectious clone constructs and the finding of a low pathogenic field virus also harboring deletion at the same position showed that this unique deletion was not directly related to virulence (Li et al., 2010, Zhou et al., 2009).

1.18. Lena and SU1-bel

The genetic diversity between the Type 1 subtypes has lead to the question of these as source of differences in pathogenicity between these subtypes. In the winter of 2007, a Belarusian farm confirmed positive for PRRSV experienced abortions, birth of mummified, dead and weak piglets, high mortality rate before waning, respiratory disorders and mortality (up to 70 %) in growing pigs (Karniychuk et al., 2010). PRRSV was isolated from lung tissues obtained from weak-born piglets and from the ORF5 and ORF7 sequences the virus were classified to belong to PRRSV Type 1 subtype 3 and the virus strain was designated 'Lena' (Karniychuk et al., 2010). The complete genome of Lena has been sequenced and alignments of Lena with the Type 1 subtype 1 reference sequence, LV, showed variations over the entire genome (Van Doorsselaere et al., 2012).

Three experimental infection studies with subtype 3 PRRS viruses have been performed (Karniychuk et al., 2010, Morgan et al., 2013, Weesendorp et al., 2013). Two of these studies were conducted using the Lena strain and Type 1 PRRS viruses belonging to subtype 1 for comparison (Karniychuk et al., 2010, Van Doorsselaere et al., 2012, Weesendorp et al., 2013). The study by Karniychuk (2010) showed that Lena induced high fever, lasting for several weeks, viremia detectable for 4 weeks PI, and fibrinous pleuropneumonia, pericarditis, and peritonitis were observed gross lesions (Karniychuk et al., 2010). The second infection study with Lena showed less severe clinical signs compared to the first study, however the immune response from pigs inoculated with Lena showed differences compared to PRRSV Type 1 subtype 1 viruses (Weesendorp et al., 2013). The third experimental infection study with a PRRSV Type 1 subtype 3 virus SU1-bel (but distinct from Lena) was performed (Morgan et al., 2013), and the results indicated increased clinical and pathological effects of the SU1-bel strain caused by an enhanced inflammatory immune response (Morgan et al., 2013).

From these studies it is evident that PRRSV Type 1 subtype 3 is able to induce severe disease in pigs (Karniychuk et al., 2010, Morgan et al., 2013, Weesendorp et al., 2013).

1.19. Diagnostic tools

Diagnostic tools for detection of PRRSV are very important since diagnosis of PRRSV on the basis of clinical signs in the field is very difficult due to variations in the signs of infection (Botner, 1997). The most common diagnostic tools for the diagnosis of PRRSV are immunoperoxidase monolayer assay (IPMA) (Wensvoort et al., 1991, Botner et al., 1994), enzyme-linked

immunosorbent assay (ELSIA) (Sorensen et al., 1998), and real-time RT-polymerase chain reaction (real-time RT-PCR) (Kleiboeker et al., 2005, Balka et al., 2009, Wernike et al., 2012).

The IPMA method relays on measuring the binding of PRRSV antibodies in the sample serum to control virus present on the cell surface. At the National Veterinary Institute, DTU, Denmark, the IPMA plates are prepared by inoculating Marc-145 cells with a control virus (Type 1 or Type 2 PRRSV) for approximately 24 hours following fixation in absolute ethanol. The fixated cells then incubate with control and test serum in different dilutions. If the test serum contains antibodies against PRRSV it will bind to the viruses present on the cell surface of the fixated cells. Peroxidase conjugated rabbit anti-swine immunoglobulin (IgG) is then added to the cells and will if present bind to the PRRSV antibodies attached to the cell surface. The IPMA is developed by the addition of H_2O_2 in ethylcarbazole and the plates can be interpreted using a light microscope (Botner et al., 1994, Wensvoort et al., 1991).

The ELISA method also relies on the detection of antibodies against PRRSV. At the National Veterinary Institute, DTU, Denmark, an 'in house' blocking ELISA is used for the detection and discrimination of PRRSV antibodies in serum samples (Sorensen et al., 1998). Microplates coated with Type 1 or Type 2 PRRSV antigen (separate plates) are added sample serum. If the test serum contains antibodies against the PRRSV antigen it will bind to the coated wells. Then biotinylated anti-PRRSV immune serum IgG is added which will only bind to the PRRSV antigens if the test serum was negative for PRRSV antibodies, since the presence of antibodies in the test serum will saturate the antigen-coat and hence no anti-PRRSV immune serum IgGs will be able to bind. To monitor this, peroxidase conjugated Avidin is added which will also only bind if the test serum was negative. The results of the ELSIA are visualized by a colorimetric reaction and the optical density (OD) is measured. OD% values above 44 means that the test serum was negative for PRRSV antibodies. If the ELISA for Type 1 and Type 2 are run parallel, and the outcome was positive for PRRSV (OD% < 44) the EU/US ratio can determined the genotype of the virus. EU/US ratios above 1.9 indicate Type 2 PRRSV and EU/US ratios below 1.3 indicate Type 1 PRRSV (Sorensen et al., 1998). A disadvantage with PRRSV antibody serological tests are that they do not discriminates between seropositive animals which have cleared PRRSV infection and carrier animals or whether the pigs are still viraemic (Oleksiewicz et al., 2001). However, high IPMA titers generally may indicate an acute infection (Botner, 1997).

A common method for the detection of virus is real-time PCR. Several versions and chemistries have been developed and utilized for the detection of PRRSV (Balka et al., 2009, Kleiboeker et al.,

2005, Kubista et al., 2006, Oleksiewicz et al., 1998). The advantage using real-time PCR is that it is fast, sensitive, and can be performed in high throughput (Kubista et al., 2006). The real-time PCR is performed on DNA, single-stranded (ssDNA) or double-stranded (dsDNA), and uses two primers that flank the nucleotide sequence to be amplified, the four deoxynucleotide triphosphates (dNTP), and a heat-stable polymerase (Kubista et al., 2006). The real-time PCR is performed by temperature cycling; first step is carried out at a high temperature to separate/melt the ds-DNA, then the temperature is lowered to let the primers anneal to the template DNA, and finally the temperature is set to the preferred temperature of the polymerase used (Kubista et al., 2006). During the real-time PCR a florescence signal is generated, from the incorporation of fluorescence either from fluorescent dyes or reporter fluorescent molecules attached to the probes or in some chemistries also one of the primers. The level of the fluorescent signal is measured in real-time at each temperature cycle and reflects the amount of DNA copies generated during the PCR reaction (Kubista et al., 2006). Since PRRSV has an RNA genome, an initial step of reverse transcription (RT) needs to be carried out for the generation of a DNA copy (cDNA) of the RNA. This reaction is typically carried out at 50-60 ºC for 30 minutes by a reverse transcriptase. The primer initiating the cDNA synthesis may be one of the PCR primers (reverse primer) or a specifically designed RT primer which could be gene specific, poly(dT) or a mix of short random primers. Hence, PCR and real-time PCR using an RNA template is referred to as RT-PCR or real-time RT-PCR. The RT step can be in a separate reaction or in the same tube as the PCR the latter is referred to as OneStep RT-PCR/real-time RT-PCR. Practical problems with the real-time RT-PCR method for detection of PRRSV is the high diversity of PRRSV since the sensitivity of the assay rely on the nucleotide match between the template PRRSV RNA and the primers and probes used. Due to the high diversity of PRRSV, it is difficult to design conserved primers matching all PRRSV genomes. A solution to this key problem which more recently has become prevalent is the use of mixture of several primer pairs and probes to cover a broader range of variant templates. The strategy can also be exploited in the design of assays that can discriminate between the two PRRSV genotypes for the simultaneous detection and typing of PRRSV. Another strategy is simply to run more than one assay. The real-time RT-PCR assay presently used for detection of PRRSV at the National Veterinary Institute, DTU, Denmark, employs the Primer Probe Energy Transfer (PriProET) chemistry (Balka et al., 2009). This system relays on respectively flourophor labeled reverse primer and probe with sense orientation. The probe is a short oligo complementary to a part in the region flanked by the forward and reverse primers. The reverse primer is labeled at its 5'-end and the probe at its 3'end. When the sense probe

hybridizes to the revere strand fluorescence is emitted since the flourophor on the reverse strand excites the flourophor on the probe. A melting curve can be collected following the PCR reaction and represent the matching of the probe to the amplicon hence genotyping of PRRSV can be determined.

Conventional PCR can also be used for the detection of PRRSV. It uses a forward and a reverse primer and the PCR product is visualized by agarose gel electrophoresis. The DNA products obtained from conventional PCR can be used for sequencing, where the nucleotide composition and order will be determined.

PRRSV can be isolated following growth on either PAM or Marc-145 cells (Kim et al., 1993, Wensvoort et al., 1991). Propagation of Type 2 PRRSV cannot take place in PAMs so a common cell line for propagation of Type 2 PRRSV is the Marc-145 cells and Type 1 PRRSV prefers PAMs however adaption to Marc-145 cells is possible (Botner et al., 1994, Botner, 1997). Cytopathic effect (CPE) is visible 2-3 days following inoculation and indicates PRRSV propagation in the cells. The cells can be immunohistochemically stained with a monoclonal antibody (SDOW17) or similar to confirm the presence of PRRSV (Fig. 1.5).

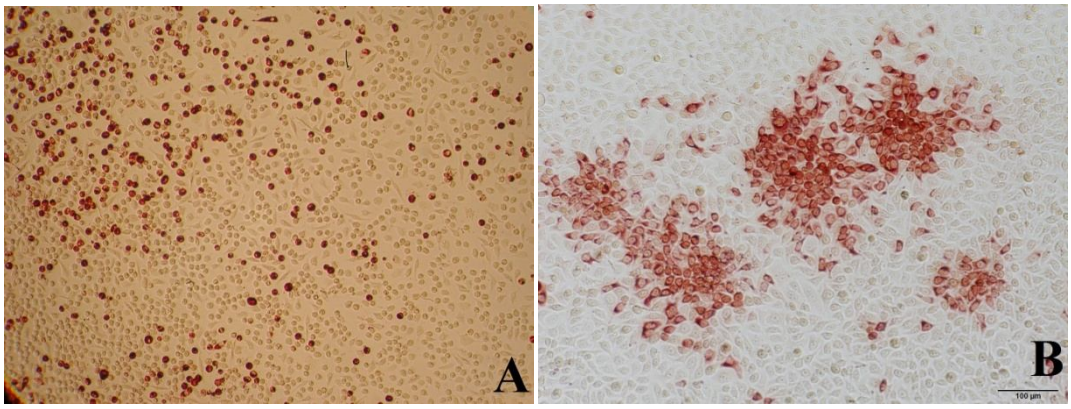

Fig. 1.5. Immunohistochemically stained PAM and Marc-145 cells. *Panel A: PAM inoculated with Type 1 PRRSV. Panel B: Marc-145 cells inoculated with Type 2 PRRSV. Images are kindly provided by A. Botner and J. Nielsen.*

1.20. Vaccines

Several vaccines have been produced to combat PRRSV (Murtaugh and Genzow, 2011). Both attenuated live vaccines (e.g. Ingelvac® PRRS MLV and Porcilis® PRRS) and inactivated vaccines

(e.g. Progressis® and PRRomiSe®) have been developed (Murtaugh and Genzow, 2011). Vaccination against PRRSV has generally not been very successful, partially because of the rapid mutation rate and evolution of the virus, lack of cross-protection, and weakness in inducing the 'correct' immune response in the animal (Nauwynck et al., 2012). Nevertheless, protection provided by the attenuated live vaccines is better than that from inactivated vaccines, however the protection from the attenuated live vaccine still rely on homologous situations (Murtaugh et al., 2002, Labarque et al., 2003).

A very serious problem with the use of attenuated live vaccines is the risk of reversion to high virulence. This scenario was seen following a vaccination program with the Ingelvac PRRS MLV that led to an epidemic of Type 2 PRRSV in the previously PRRSV Type 2 free country, Denmark (Botner et al., 1997, Madsen et al., 1998, Nielsen et al., 2001).

In 2010 the EU project: New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia (PoRRSCon) were started by the initiative of 14 partners from Europe and Asia. The final goal of the EU project is to develop new generation, efficacious and safe maker vaccines that can be adapted to temporary changes and geographical differences.

Manuscript I

A fast and robust method for full genome sequencing of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Type 1 and Type 2

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(Submitted for publication)

Abstract

PRRSV is a positive-sense RNA virus with a high degree of genetic variability among isolates. For diagnostic sensitivity and vaccine design it is essential to monitor PRRSV genetic diversity. However, to date only a few full genome sequences of PRRSV isolates have been made publicly available. In the present study, fast and robust methods for long range RT-PCR amplification and subsequent next generation sequencing (NGS) were developed and validated on nine Type 1 and nine Type 2 PRRSV viruses. The methods generated robust and reliable sequences both on primary material and cell culture adapted viruses and the protocols performed well on all three NGS platforms tested (Roche 454 FLX, Illumina HiSeq2000, and Ion Torrent PGM™ Sequencer). These methods will greatly facilitate the generation of more full genome PRRSV sequences globally.

Keywords

PRRSV, full genome sequencing, next generation sequencing, Illumina HiSeq2000, Roche 454 FLX, Ion Torrent PGM sequencer

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS), a potent viral disease of pigs, has a major impact on the health and welfare of pigs throughout the world. PRRSV causes reproductive failures in sows and gilts and respiratory diseases in growing pigs (Collins et al., 1992; Terpstra et al., 1991; Wensvoort et al.; 1991). Different PRRSV strains have been claimed to differ in virulence, but the underlining molecular determinants are still unknown despite decades of research.

PRRS virus belongs to the *Arteriviridae* family, within the order *Nidovirales* (Cavanagh, 1997). It is a small enveloped virus containing a positive-sense single-stranded RNA genome with a 5' cap and a polyadenylated 3'-end (Benfield et al., 1992; Meulenberg et al., 1998). The genome is 15-15.5 kb in length and encodes 10 ORFs including the recently discovered ORF5a (Firth et al., 2011; Johnson et al., 2011; Snijder and Meulenberg, 1998; Wu et al., 2001). Based on nucleotide sequence comparison of European and North American isolates, PRRSV is divided into two genotypes, Type 1 and Type 2 respectively, with only 50-60 % nucleotide identity (Allende et al., 1999). Furthermore, Type 1 PRRSV genotype has been found to be more diverse, and can be categorized into at least four subtypes, where the protogenotype 1, the Lelystad virus (Genbank: M96262), belongs to subtype 1 (Stadejek et al., 2002; Stadejek et al., 2006; Stadejek et al., 2008).

Most of the molecular analyses of the PRRSV genome have been performed on the genes coding for the structural proteins (ORF2-7) only. Of the seven structural proteins, glycoprotein 5 (GP5) encoded by ORF5 and the nucleocapsid (N) protein encoded by ORF7 have been the most examined. GP5 has been shown to contain a B-cell neutralizing epitope and is regarded as the most immunogenic viral protein, whereas the N protein has shown size polymorphism (Ansari et al., 2006; Oleksiewicz et al., 2002; Ostrowski et al., 2002; Stadejek et al., 2002). Previous reports suggested that the length of ORF7 could be used as a subtype maker since its length varied between Type 1 strains originating from Eastern Europe (Stadejek et al., 2002; Stadejek et al., 2006; Stadejek et al., 2008). However, recently a PRRSV isolate from Slovakia encoded a 132 amino acid N protein (compared to 128 amino acids N protein in the Lelystad virus), but otherwise resembled the subtype 1 PRRSV (Jackova et al., 2012). These findings emphasized that the diversity of PRRSV are more complex than initially anticipated and that examination of small genetic regions is not sufficient for understanding PRRSV sequence heterogeneity. This is further supported by the emergence of a highly pathogenic strain of PRRSV in China in 2006, which was highly similar to other Type 2 PRRSV strains in ORF5 and ORF7, but had indeed unique feature differences in other genes (An et al., 2010; Tian et al., 2007). Until now, only few full genome sequences of European

PRRSV isolates have been characterized (Darwich et al., 2011) and similarly little is known on the diversity of European Type 2 strains circulating in Europe. To accelerate the generation of full genome sequences of PRRSV, application of next generation sequencing (NGS) technology is the method of choice. In recent years the cost of using this technology has been reduced substantially and hereby making it more accessible (Glenn, 2011). The aim of the present study was to develop and validate a method for robust and reliable full length sequencing of PRRSV Type 1 and 2 based on long range PCR performed on viral RNA extracted from both primary material and cell culture isolates.

2. Material and methods

2.1. Sample material

Samples included in this study were collected in Danish pig farms during the years 1992 to 2012. Details on the viruses and their origin are listed in Table 1. Some of the viruses were proliferated in Marc-145 cells or porcine alveolar macrophages (PAM) following the general procedure (Kim et al., 1993; Wensvoort et al., 1991). The ESP-1991-Olot91 "third passage MA-104" isolate was obtained from Dr. Luis Enjuanes (Department of Molecular and Cell Biology, CSIC, Madrid, Spain). One further passage in Marc-145 was carried out prior to sequencing.

2.2. Viral RNA purification

Viral RNA was purified from serum, lung tissues or cell culture supernatants. Lung tissue was prepared as a 5 % homogenate in RLT buffer (RNeasy® Mini Kit, QIAGEN, cat. no. 74106) with 1 % β-mercaptoethanol (Sigma-Aldrich, cat. no. M3148). One steel bead (QIAGEN, cat. no. 69989) was added to each sample and the samples were homogenized in a Tissuelyser II (QIAGEN, cat. no. 85300) for three minutes at 30 Hz. The homogenate was centrifuged for three minutes at 12,000 x *g* 600 µl lung tissue homogenate was mixed with an equal volume of 70 % ethanol by pipeting and then transferred to an RNeasy® Mini column. The rest of the purification procedure was performed as described by the manufacturer's instructions for purification of total RNA from animal tissues with RNeasy Mini Kit (QIAGEN). RNA from serum and cell culture supernatant was purified using QIAamp® Viral RNA Mini Kit (QIAGEN, cat. no. 52906) using the conditions recommended by the manufacturer. The RNA was stored at -80°C until use.

2.3. Screening for PRRSV with real-time RT-PCR

PRRSV positive samples we selected for sequencing by screening of purified RNA using a modification of the Primer Probe Energy Transfer RT-PCR (PriProET-RT-PCR) OneStep real-time RT-PCR assay previously described (Balka et al., 2009).

2.4. cDNA synthesis

Full-length cDNA synthesis was performed with SuperScript® III First-Strand Synthesis System (Invitrogen, cat. no. 18080-51) using 3'-end poly(dT) reverse transcription (RT)-primer: $^{5'}$CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT T(T)$_{38}{}^{3'}$ (Nielsen et al., 2003). The method for the synthesis of full-length cDNA was as previously described for pestiviruses (Rasmussen et al., 2008; Rasmussen et al., 2010). In short, 8 µl of purified RNA (approx. 0.7 µg) was mixed with 1 µl of 10 µM RT-primer and 1 µl of 10 nM dNTPs. The mixture was incubated for 5 minutes at 65°C in a thermal cycler with heated lid and then placed on ice for at least 1 minute. A second mixture containing 2 µl 10x RT buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µl 40 U/µl RNaseOUT™, and1 µl SuperScript III RT™ was prepared and mixed gently with the RNA/primer mixture. The cDNA synthesis was performed in a thermal cycler with heated lid at 50°C for 90 minutes followed by incubation for 5 minutes at 85°C to inactivate the reverse transcriptase. To collect the samples a brief centrifugation step was applied and the cDNA was treated with 1 µl of 2 U/µl *E. coli* RNase H for 20 minutes at 37°C to remove the RNA template. The cDNA was stored at -20°C until use.

2.5. Primers for long range PCR amplification

All the primer sequences used for long range PCR amplification are listed in Table 2. Each primer sequence was aligned against the sequence of the two PRRSV proto-genotypes, Lelystad virus (M96262) or VR2332 (U87392.3) using CLC Main Workbench v.6.6.2 (CLC BIO, Aarhus, Denmark). The primer sequences were adjusted to match the sequence of the two proto- genotype strains.

2.6. Long range PCR amplification for sequencing

PCR amplifications were performed with AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen, Cat. no. 12346-086). For amplification of PCR products covering the PRRSV genome in 4 fragments, 2 µl of cDNA template was mixed with 5µl 10x AccuPrime™ PCR buffer I, 1µl 10

µM specific forward primer (Table 2), 1 µl 10 µM specific reverse primer (Table 2), 0.5 µl AccuPrime™ *Taq* DNA Polymerase High Fidelity, and nuclease free water (AMRESCO®, cat. no. E476) in a final reaction volume of 50 µl. For amplification of PCR products covering the PRRSV genome in 2 fragments, 1 µl of AccuPrime™ *Taq* DNA Polymerase High Fidelity was used. The PCR amplification was carried out in a T3 Thermocycler (Biometra) under the following conditions: 94°C 15 seconds, [45 cycles: 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 60 seconds per kb amplification], 68°C for 2 minutes per kb amplification, hold at 4°C. The PCR products were analyzed by agarose gel electrophoresis using E-gel® 0.8 % agarose gels (Invitrogen, cat. no G501808). Five µl of PCR product mixed with 7 µl 1:40 TrackIt™ loading buffer (Invitrogen, cat no. 10482-028) were loaded onto the gel. 10 µl 1 kb Plus DNA ladder as marker (Invitrogen, cat. no. 10787-018) was used as size marker. To remove primers and buffers, the PCR products were purified using Roche's High Pure PCR Product Purification kit (cat. no. 11 732 676 001) following the manufacturers protocol, with the exception that elution was done in 30 µl nuclease free water (Amresco, cat. no. E476).

2.7. Full genome sequencing

Three different platforms were used for next generation sequencing. For the sequencing of PCR amplicons covering the full genome of PRRSV in two fragments, 2.7 µg in total of equimolar concentration were sent to LGC Genomics GmbH (Berlin, Germany) for sequencing on the platform Roche 454 FLX. The two samples were sequenced at separate occasions on a 1/16 Roche/454 Pico Titer Plate pooled together with 10 and 11 other libraries prepared from PCR amplicons of another virus with approximately the same size. The sequencing of PCR amplicons covering the PRRSV genome in four fragments was performed on the Illumina® HiSeq2000 and Ion Torrent PGM™ sequencer platforms. The four PCR products representing one PRRSV genome were pooled in equimolar quantities to a final amount of 1 µg. The sequencing on the platform Ion Torrent PGM™ sequencer was carried out by the DTU in-house facility (DTU Multi-Assay Core (DMAC), Technical University of Denmark). An Ion Torrent PGM™ chip 316 was used for 32 libraries of approximately the same size. Sequencing on Illumina® HiSeq2000, took place at ARK Genomics (The Roslin Institute, University of Edinburgh, Scotland, UK). A total of 22 libraries of the same genome size were pooled and sequenced on one channel flow cell. The preparation of these libraries was performed following the Illumina® TruSeq™ DNA Sample Preparation v2 Kit A and B with minor exceptions (Illumina®, cat. no. FC-121-2001-1 and FC-121-2002). Instead of

purifying the samples using Agencourt AMPure XP beads as stated in the manufactures protocol, column purifications was performed using MinElute PCR Purification Kit (QIAGEN, cat. no. 28004).

2.8. 5'-RACE

For Type 2 viruses amplified using the forward primer 'Fragment-A-US-FW' (Table 2), the 5' end nucleotides were not amplified and therefore 5' Rapid Amplification of the cDNA Ends was performed to complete the sequences. This was performed using 5' RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, cat. no. 18374-058) according to instructions from the manufacturer. In short, one gene-specific RT-primer was designed for the cDNA synthesis, one for the first PCR amplification and one for the final nested PCR using Primer3Plus web utility (Untergasser et al., 2007). The nested PCR products of approximately 500 bp were purified directly from the reactions using Roche's High Pure PCR Product Purification kit (Roche, cat. no. 11 732 676 001) or they were excised from E-gel 0.8% agarose gels and extracted using MinElute Gel Extraction Kit (QIAGEN, cat. no. 28604). The final RACE products were cloned into pCR®4-TOPO® vectors and transformed into *E. coli* One Shot® TOP10 competent cells following the protocol for TOPO® TA Cloning® Kit for Sequencing (Invitrogen, K4575-02). Sequences were obtained with cycle sequencing performed at LGC Genomics using M13 primers.

2.9. Sequence data analysis

After initial removal of adaptors and low quality sequences, the quality of the FastQC files was examined using the applicant FastQC (version 0.10.1). The reads were trimmed in regards to the FastQC report. Trimming was done by the Prinseq-lite tool and mapping of the reads was performed by the Burrows-Wheeler aligner (BWA) using the Aln algorithm for the Illumina® HiSeq2000 data and the bwasw algorithm for Roche 454 FLX and Ion Torrent PGM™ sequencer data. Coverage depth was calculated by a combination of Samtools and Bedtools.

The full length nucleotide sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log- Expectation). Phylogenetic trees of nucleotide sequences were constructed using Maximum Likelihood Phylogeny with the following parameter settings: Starting tree: Neighbor Joining, Substitution model: HKY, Transition /transversion ratio = 2.0, Rate variation: none, Estimate substitution rate parameter(s) = yes, Estimate topology = yes. All the phylogenetic analyses were carried out using CLC Main Workbench v.6.6.2 (CLC BIO, Aarhus, Denmark).

3. Results

3.1. Specificity of the PCR products

The long range PCR amplicons generated in this study covered the complete PRRSV genome in two or four fragments, with sequence overlap of 270-677 bp between the fragments (Table 2). To confirm the size, the purified PCR products were run on a 0.8 % agarose gel (Fig. 1). Most of the PCRs produced a single amplicon with the expected size, however, in few cases an unspecific band was in excess (Fig. 1, panel A, lane 4). In other cases, the PCR product of interest appeared in equal amount as unspecific products (Fig. 1, panel B, lane 9). In those cases with ambiguous products, the PCR amplicon of interest was gel extracted prior to sequencing.

3.2 Sequence quality and trimming

To evaluate the quality of the NGS data, adaptors and low quality sequences were first removed prior to further analysis with the FastQC application. This application helped visualize the quality of the reads by examining different parameters such as the distribution of the nucleotides in each read, read lengths, and calculated the Phred Quality Score of each nucleotide in each read. The Phred Quality Score described the probability of incorrect base calls and allowed to compare the efficacy of the different sequencing platforms. A Phred Quality Score of 30 (Q30) is equivalent to the probability of an incorrect base call in 1 out of a 1,000 times, which gives the base-call accuracy of 99.9 % (http://www.illumina.com/Documents/%5Cproducts%5Ctechnotes%5Ctechnote_Q-Scores.pdf). A box plot of the Phred Quality Score distribution is shown in Fig. 2 and in supplementary material Fig. 1. The FastQC report obtained for Roche 454 FLX sequencing showed a decline in 3' end reads quality and thus the major read lengths were trimmed from 592 bp to 450 bp. The box plot of the Phred Quality Score distribution is shown in Fig. 2A- B. The average Phred Quality Score of the HiSeq2000 Illumina data was Q36.5 and which was highly acceptable. Although, a small decrease in the Phred Quality Score was observed at the 5' end of the reads (Fig. 2, panel C- D, and supplement Fig. 1A-B), the mean Q values were still above Q30, thus there was no reason for further trimming of these data. The majority of the full genome sequences of PRRSV were generated using the Ion Torrent PGM sequencer. The analysis of these data by FastQC, showed "warnings" in both the 5' and 3' ends of the reads with regard to the distribution of the four nucleotides. Thus, the reads were trimmed to a major length distribution of 170 bp to reach a minimal Phred Quality Score of Q20 for both the 5' and 3' ends. This strategy resulted in a mean

Phred Quality Score of Q24 or Q25 (Fig. 2, panel E-F and supplement Fig. 1C-L), which gave an acceptable base call accuracy between 99-99.9 %. An overview of the average Phred Quality Scores and major read lengths is listed in Table 3.

3.3 Sequence output and coverage

The data output varied among the different NGS platforms used, with the highest yield for the Illumina HiSeq2000 platform. In Table 3, total reads and mapped reads are listed for all samples. The amount of mapped reads in percent ranged from 23.8 % to 99.2 %. The outcome of mapped reads below 40 % may be result of low purity of the PCR amplicons in the sample or low quality of the library preparation. The distribution of the mapped reads throughout the genomes is shown in Fig. 3 and supplementary material Fig. 2 as a depth-of-coverage graph. Peaks with a substantially larger depth of coverage are present in all graphs. These peaks correspond to the overlap in sequence of the PCR amplicons. Variations in the coverage depths were seen between the PCR amplicons from the same sample, which indicate different quality and purity of the PCR amplicons or imprecise preparation of equimolar pools. In some cases regions without coverage was observed (Fig. 3) by that indicating gaps compared to the reference sequence. The presence of gaps were either confirmed or rejected by cycle sequencing.

The complete full genome sequences had the lengths of 14,876-15,098 and 15,342-15,408 nucleotides, for the Type 1 and Type 2 viruses, respectively (excluding the poly(A)-tail). Phylogenetic trees of the obtained full length sequences are shown in Fig. 4. The clustering of the viruses in the trees based on the full genome sequences were as expected and resembled the clustering of the isolates when the tree was based on ORF5 sequences only (data not shown).

4. Discussion

The main focus of this study was to develop fast and robust methods for full length sequencing of PRRSV directly from infected tissues using next generation sequencing platforms. The protocols generated sequences with the expected lengths and quality as illustrated by the preliminary phylogenetic analysis which showed the expected clustering of the analyzed isolates. Further analyses of the full genome sequences are in progress.

The protocols described in the present study were based on the generation of long range PCR products using a high fidelity polymerase which has a low error-rate. This high fidelity polymerase

is a mixture of recombinant *Taq* DNA polymerase, *Pyrococcus* species *GB-D* polymerase, and Platinum *Taq* Antibody which results in a nine-fold increase in fidelity. Furthermore, this enzyme blend is effective over a wide range of target sizes up to 20 kb with some optimization (http://tools.invitrogen.com/content/sfs/manuals/accuprimetaqhifi_man.pdf). The protocol describes the sequencing of PCR amplicons covering the PRRSV genome in 2 and 4 fragments. Sequencing from two fragments has the advantage, besides the reduced labor handling only two amplicons, that only one sequence overlap will occur and less mismatch/bias from the primer sequencing will be incorporated in the PCR amplicons as only two primer pairs are used in the production of two PCR amplicons. On the other hand, the disadvantage when amplifying the PRRSV genome in two PCR fragments was that the concentration of the specific PCR amplicons often were of low concentration as they often needed to be extracted from the gel instead of direct purification from the PCR mix due to the present of unspecific products. The advantage of sequencing from PCR amplicons covering the PRRSV genome in four fragments was that the PCR products were of relatively high concentrations and specificity hence the amplicons could be purified directly from the PCR mix without the need for gel purification. The disadvantage of sequencing on four fragments is that two sites of overlapping sequences will occur. The sequence primer bias is not problematic when sequencing with the NGS technology is performed to obtain a consensus sequence for phylogenetic and amino acid analyses, but should be kept in mind if the PCR products are sequenced for quasispecies and de novo analyses.

The main advantage of this method is that it enables viral RNA extracted directly from the primary material to be used as template for the cDNA synthesis. Such an approach bypasses the needs for propagation of PRRSV in cell culture which can be tedious and time-consuming. The use of primary material for sequencing furthermore prevents the introduction of mutations caused by cell culture adaptation and maintains the original level of quasispecies distribution. This new, innovative method offers a tool for a faster and quicker production of full genome PRRSV sequences compared to previous published protocol (Darwich et al., 2011; Van Doorsselaere et al., 2011).

Since only one NGS platform is available on most institutions, the applicability of the methods described here is tempting because all three tested NGS platforms were shown to generate PRRSV sequences of satisfactory quality. The selection of the optimal platform should be based on a variety of considerations including price, run time, read lengths, depth of coverage (yield data output). In the present set-up, the coverage depth was considerably higher for the Illumina HiSeq2000

compared to Roche 454 FLX and Ion Torrent PGM sequencer. Since PRRSV has a relative short genome of 15 kb, the coverage depth obtained in this study from all three NGS platforms was satisfying. The run time for Illumina HiSeq2000 was 11 days compared to the run time for Roche 454 FLX and Ion Torrent PGM sequencer of 10 and 2 hours respectively. The short run times gives these latter two platforms an advantage in applications where a fast result is crucial. For *de novo* assembly, read lengths should be a factor to consider. In this study, 37 bp read lengths were obtained from the Illumina and reads from the Roche 454 FLX and Ion Torrent PGM sequencer were trimmed to major read lengths of 450 and 170 bp, respectively. The reads were mapped using a reference sequence and therefore the short read length was not a problem.

In conclusion, the long-range PCR protocols of the present study offers a robust, cheap and fast method for the generation of full length PRRSV sequences directly from serum, tissues and cell cultures. The results of this work will be an added value for the development of new diagnostics and vaccine designs.

5. Acknowledgements

Dr. Anette Botner is gratefully thanked for the provision of PRRSV cell culture isolates.

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 245141 (New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia (PoRRSCon) coordinated by Prof. H. Nauwynck) and the COST Action FA902: Understanding and combating porcine reproductive and respiratory syndrome in Europe (EuroPRRS.net).

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Table 1. Overview of sample origin and material.

Virus sample	Year of isolation	Genotype	Material	Origin
DK-1992-PRRS-111_92	1992	Type 1	PAM isolate	Denmark
DK-2010-10-10-3	2010	Type 1	PAM isolate	Denmark
DK-2011-05-11-14	2011	Type 1	PAM isolate	Denmark
DK-2011-05-23-9	2011	Type 1	PAM isolate	Denmark
ESP-1991-Olot91	1991	Type 1	Marc-145 isolate	Spain
DK-2003-6-5	2003	Type 1	PAM isolate	Denmark
DK-2003-7-2	2003	Type 1	PAM isolate	Denmark
DK-2008-10-5-2	2008	Type 1	Lung homogenate	Denmark
DK-2012-01-05-2	2012	Type 1	Serum	Denmark
DK-2010-10-1-2	2010	Type 2	Lung homogenate	Denmark
DK-2003-2-3	2003	Type 2	Marc-145 isolate	Denmark
DK-2004-2-1	2004	Type 2	Marc-145 isolate	Denmark
DK-2008-10-1-3	2008	Type 2	Lung homogenate	Denmark
DK-2012-01-11-3	2012	Type 2	Lung homogenate	Denmark
DK-2010-10-4-1	2010	Type 2	Lung homogenate	Denmark
DK-1997-19407B	1997	Type 2	Marc-145 isolate	Denmark
DK-2004-1-7-Pl	2004	Type 2	Marc-145 isolate	Denmark
DK-2011-030311-1	2011	Type 2	Lung homogenate	Denmark

Table 2. Primer sequences for long range PCR amplification and expected amplicon sizes

Primer name	Primer Sequence (5'-3')	Expected size (bp)
Type 1 PRRSV:		
Fragment-1-EU:		7278
5'-UTR-1-35-EU-Fw[1]	GGC GCG CCT AAT ACG ACT CAC TAT AGA TGA TGT GTA GGG TAT TCC CCC TAC ATA CAC GAC A	
ORF1-7278-EU-Rev[2]	CAG CTT CAA GGC AGT TGT CA	
Fragment-2-EU:		8178
ORF1-6920-EU-Fw[3]	CCC CTC TTT TTG AGA ATG GT	
Poly(dT)-RT[4]	CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT T(T)38	
Fragment A-EU:		3019
5'-UTR-1-35-EU-Fw[1]	GGC GCG CCT AAT ACG ACT CAC TAT AGA TGA TGT GTA GGG TAT TCC CCC TAC ATA CAC GAC	
ORF1-3019-EU-Rev[1]	CGC GGG CGC TTG AGT TCG GCA AAT T	
Fragment B-EU:		4529
ORF1-2749-EU-Fw[5]	CCT GGA CCA GCC TTT AAA TC	
ORF1-7278-EU-Rev[2]	CAG CTT CAA GGC AGT TGT CA	
Fragment C-EU:		5500
ORF1-6920-EU-Fw[3]	CCC CTC TTT TTG AGA ATG GT	
ORF2-EU-Rev[6]	GCA CAC TGA TGA GCC ATT GT	
Fragment D-EU:		
ORF2-EU-Fw[6]	CTG GCA CAG AAT TGC AGG TA	3375
Poly(dT)-RT[4]	CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT T(T)38	
Type 2 PRRSV:		
Fragment-1-US:		
Fragment-A-US-Fw[7]	GGA GGG CCA AGT CTA CTG CAC ACG A	7588
Fragment-B-US-Rev[4]	TGG TTG TGC TCA ACC GCG T	
Fragment-2-US:		7939
Fragment-C-US-Fw[4]	TCT CAG AGT TGG CGA CCC T	
Poly(dT)-RT[4]	CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT T(T)38	
Fragment-A-US:		4541/4811
Fragment-A-1-35-US-Fw	CTCGAGGGCGCGCCTAATACGACTCACTATAGGATGA CGTATAGGTGTTGGCTCTATGCCTTGGCATT	
Fragment-A-US-Fw[7]	GGA GGG CCA AGT CTA CTG CAC ACG A	
Fragment-A-US-Rev[4]	GTG TCA GGG TCA ACC ACG A	
Fragment-B-US:		3506
Fragment-B-US-Fw[4]	ATC TTG GCT GGA GCT TAC GT	
Fragment-B-US-Rev[4]	TGG TTG TGC TCA ACC GCG T	
Fragment-C-US:		5500
Fragment-C-US-Fw[4]	TCT CAG AGT TGG CGA CCC T	
Fragment-C-US-Rev[4]	ATC CTG CAC CAA AGA GAC CT	
Fragment-D-US:		2900
Fragment-D-US-Fw[4]	TTT CAG CAT CTA GCC GCC A	
Poly(dT)-RT[4]	CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT T(T)38	

[1]Modified from Fang et al. 2006; [2]Fang et al. 2006; [3]Darwich et al. 2011; [4]Nielsen et al. 2003; [5]Modified from Darwich et al. 2011; [6]Díaz et al. 2006; [7]Modified from Nielsen et al. 2003. Fw: forward primer, Rev: reverse primer.

Table 3. Read quality and output yield

Virus sample	Platform	Avg. Phred Quality Score	Major read length (bp)	Total reads[a]	Mapped reads	Mapped reads %	Bases mapped
DK-PRRS-111_92	FLX	37	450	5,679	5,633	99.2	2,534,850
DK-2010-10-1-2	FLX	29	450	13,696	13,504	98.6	6,076,800
DK-2010-10-3	Illumina	36.5[b]	37	3,151,310[c]	2,691,058[b]	85.4	99,569,146
DK-2011-05-11-14	Illumina	36.5[b]	37	5,560,952[c]	5,409,041[b]	97.3	200,134,517
DK-2011-05-23-9	Illumina	36.5[b]	37	4,810,154[c]	1,145,373[b]	23.8	42,378,801
ESP-1992-Olot91	Illumina	36.5[b]	37	3,792,606[c]	3,252,661[b]	85.8	120,348,457
DK-2003-6-5	Ion Torrent	24	170	70,875	55,844	78.8	9,493,480
DK-2003-7-2	Ion Torrent	24	170	63,656	32,276	50.7	5,486,920
DK-2008-10-5-2	Ion Torrent	25	170	75,357	51,011	67.7	8,671,870
DK-2012-01-05-2	Ion Torrent	24	170	55,378	50,193	90.61	8,532,810
DK-2003-2-3	Ion Torrent	25	170	43,915	38,252	87.1	6,502,840
DK-2004-2-1	Ion Torrent	24	170	41,549	36,998	89.0	6,289,660
DK-2008-10-1-3	Ion Torrent	25	170	74,948	55,303	73.8	9,401,510
DK-2012-01-11-3	Ion Torrent	24	170	77,893	30,033	38.5	5,105,610
DK-2010-10-4-1	Ion Torrent	24	170	52,658	41,554	78.9	7,064,180
DK-1997-19407B	Ion Torrent	24	170	56,171	53,026	94.4	9,014,420
DK-2004-1-7-Pl	Ion Torrent	24	170	65,900	62,542	94.9	10,632,140
DK-2011-030311-1	Ion Torrent	24	170	85,327	66,526	78.0	11,309,420

[a]After removal of adaptor and low quality sequences and examination by FastQC, [b]For Illumina the average Phred quality score is a mean value of the Pred quality score of R1 and R2, [c]Paired end reads

Fig. 1. 0.8 % agarose gel electrophoreses analysis of long range PCR amplicons.
A: PCR amplicons for Type 1 PRRSV. Lane 4 and 5: Frag-1-EU and Frag-2-EU, lane 7-10: Frag-A-EU, Frag-B-EU, Frag-C-EU, and Frag-D-EU.
B: PCR amplicons for Type 2 PRRSV. Lane 4 and 5: Frag-1-US and Frag-2-US, lane 7-10: Frag-A-US, Frag-B-US, Frag-C-US, and Frag-D-US.

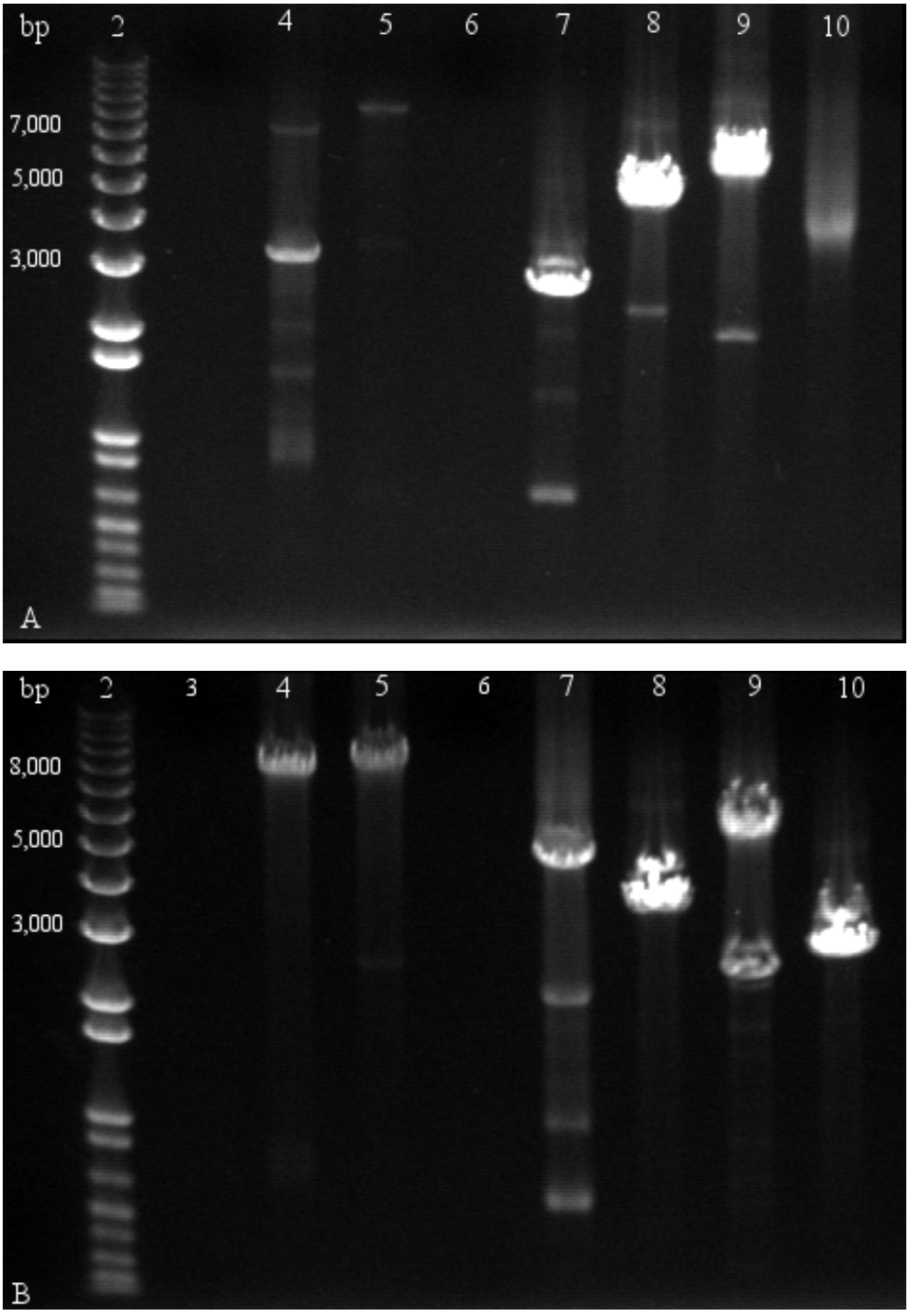


Fig. 2. Box plots of Phred quality scores generated by FastQC.
The grey box plots (box: interquartile ranges 25-75 %, whiskers: 10-90 %, and dark bar inside box: median) show the nucleotide-calling Phred quality score across all reads. A: DK-PRRS-111_92 (Roche 454 FLX), B: DK-2010-10-1-2 (Roche 454 FLX), C: DK-2011-05-11-14 (Illumina HiSeq 2000), D: Olot91(Illumina HiSeq 2000), E: DK-2011-10-4-1, (Ion Torrent PGM™ Sequencer), and F: DK-2012-01-05-2 (Ion Torrent PGM™ Sequencer).

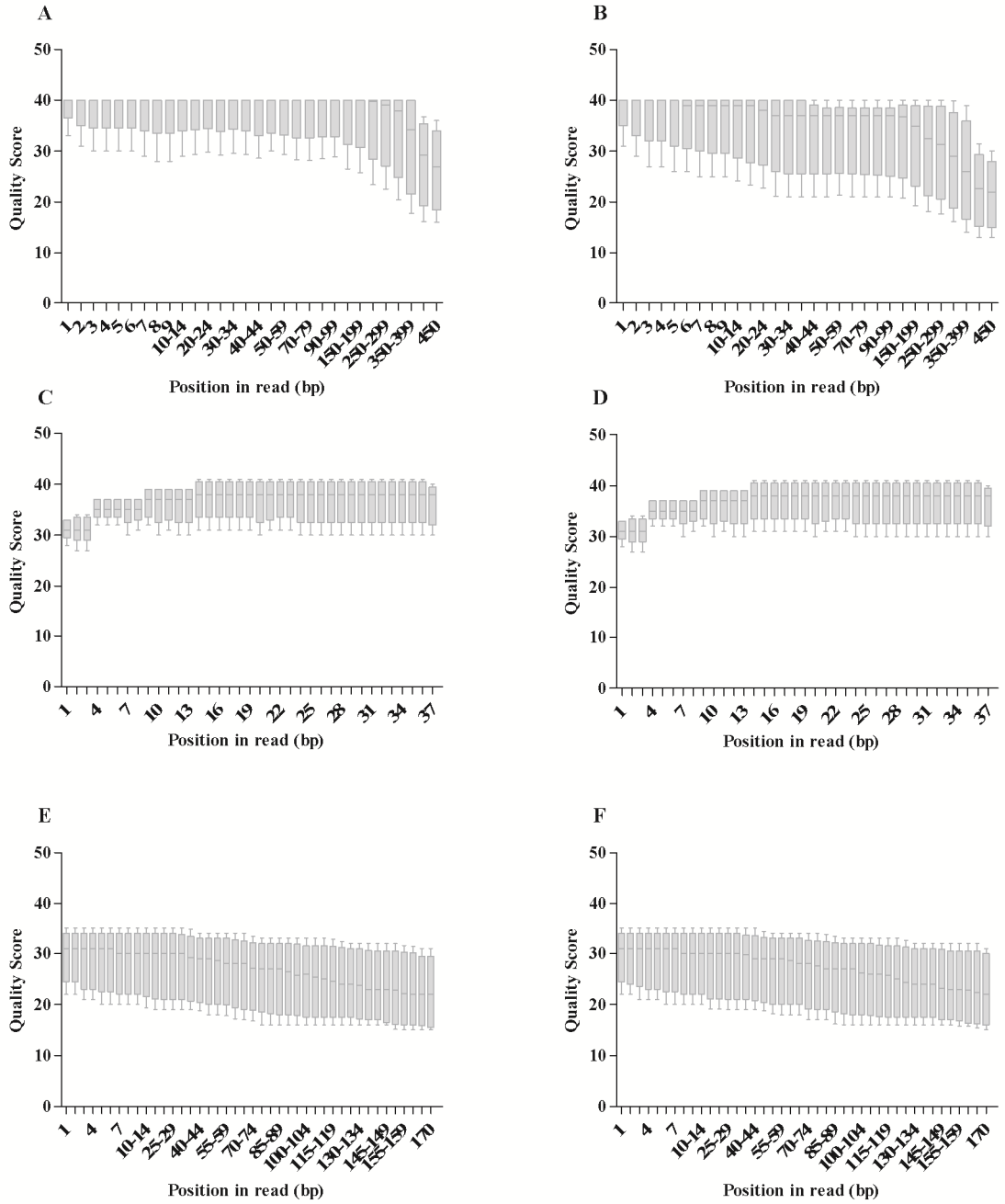


Fig. 3. Depth of coverage plot. The *x*-axes represent the coverage of every single nucleotide position in respect to the reference sequence and the *y*-axes represent the sequence depth of every single nucleotide. (A) DK-PRRS-111_92, Roche 454 FLX, (B) DK-2010-10-1-2, Roche 454 FLX, (C) DK-2011-05-11-14, Illumina HiSeq 2000, (D) Olot91, Illumina HiSeq 2000, (E) DK-2011-10-4-1, Ion Torrent PGM™ Sequencer, and (F) DK-2012-01-05-2, Ion Torrent PGM™ Sequencer.

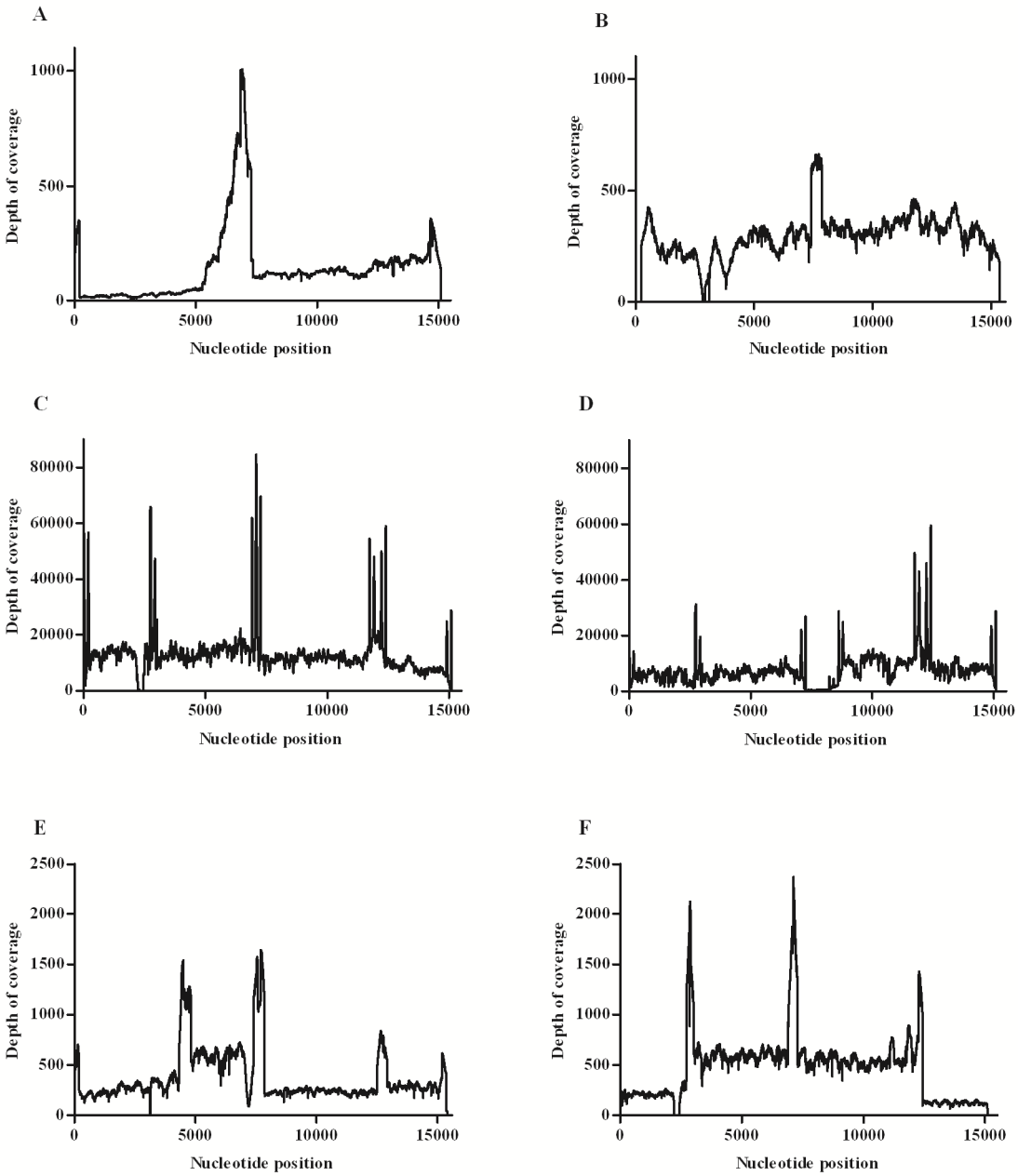
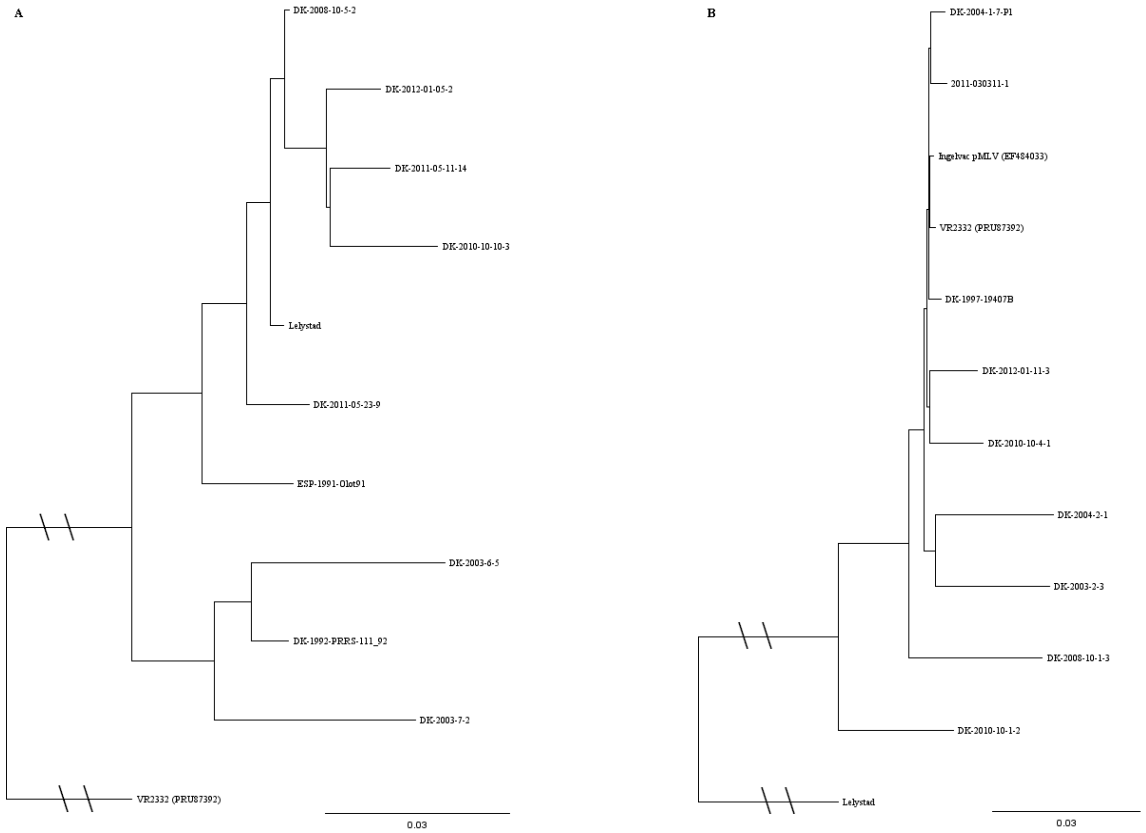
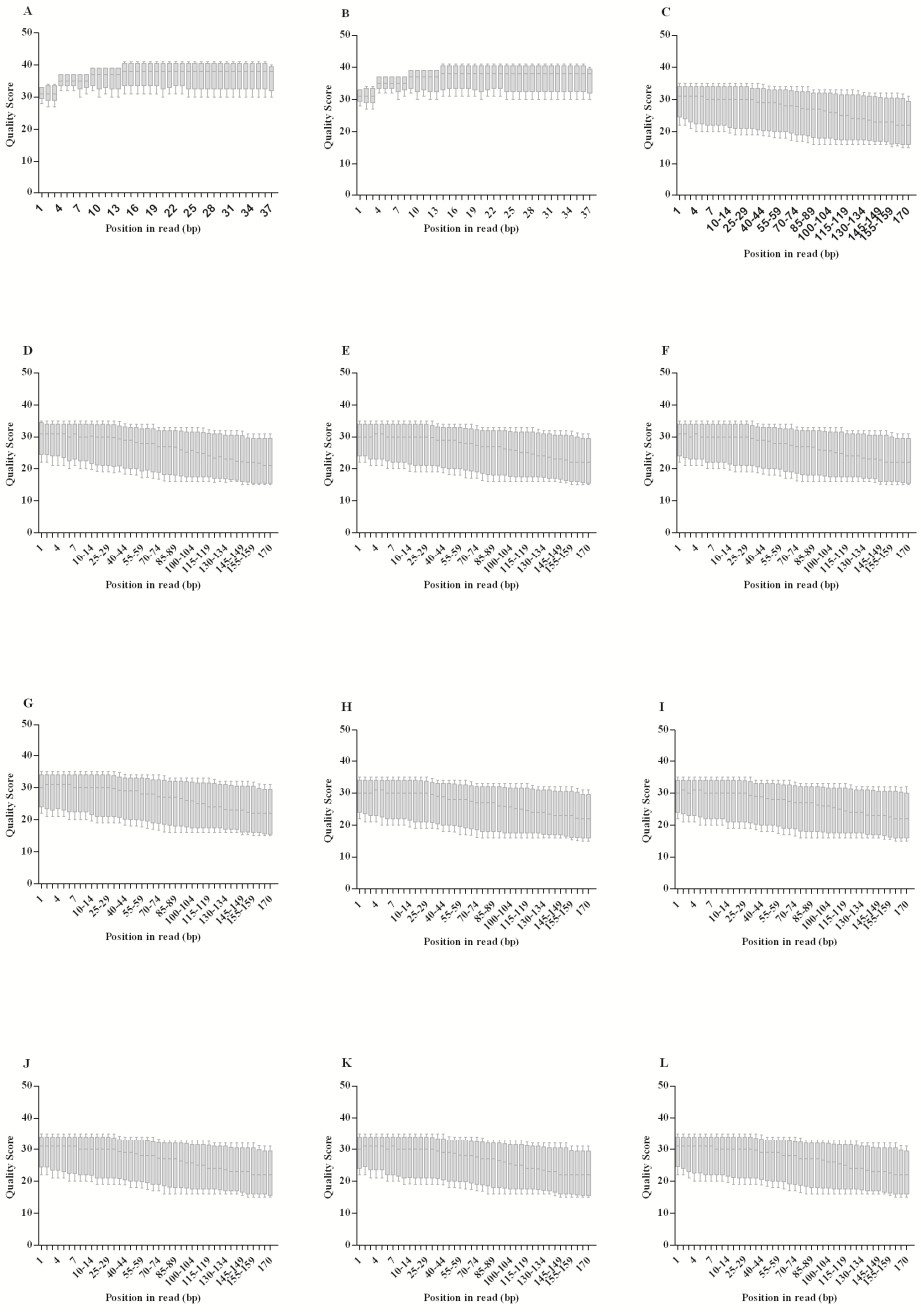


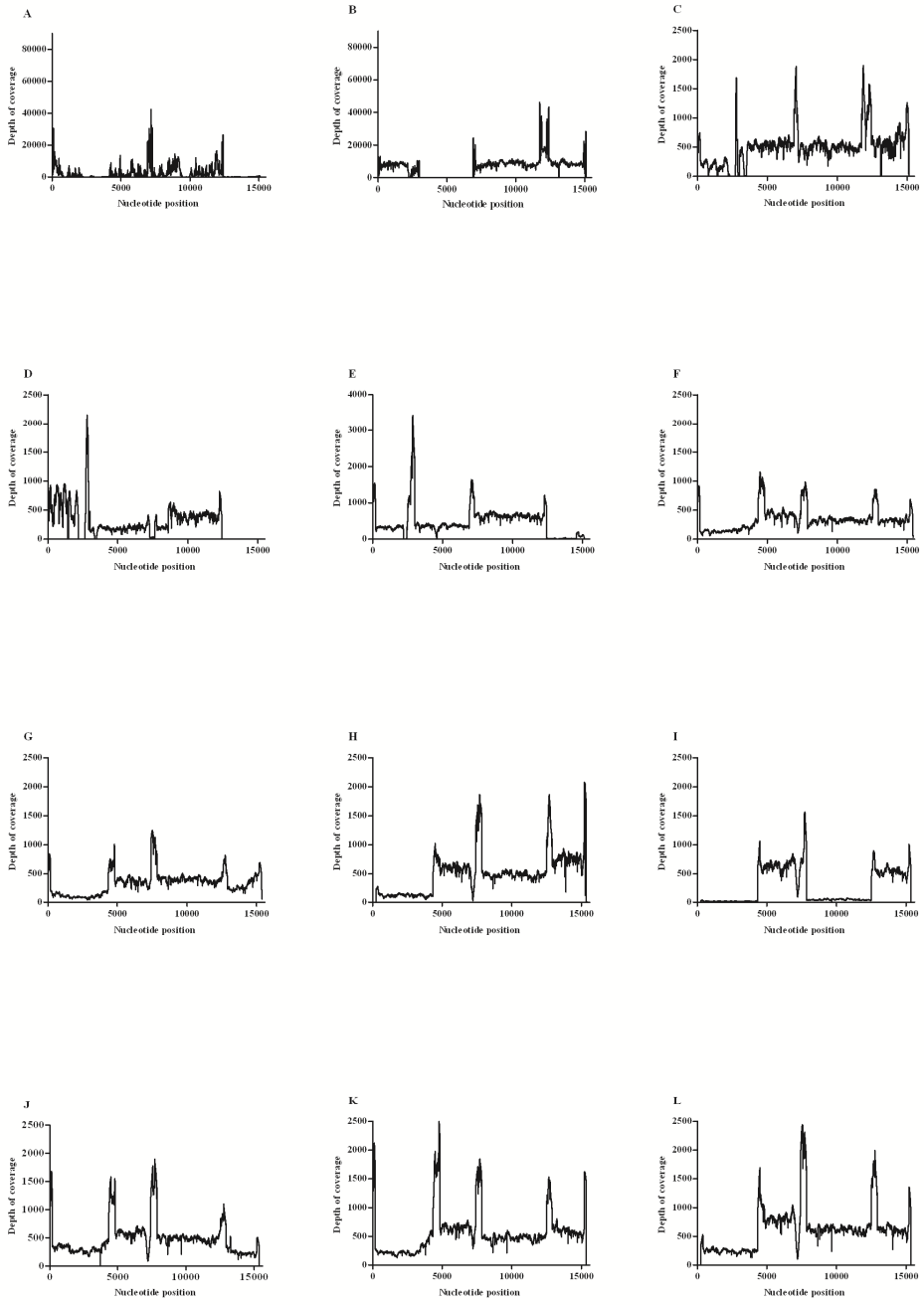
Fig. 4. Phylogenetic trees of full length genomes. Panel A: Type 1 PRRSV viruses, panel B: Type 2 PRRSV viruses. Scale bar represents 3 nucleotide changes per hundred.



Supplementary Fig. 1. Box plots of Phred quality scores generated by FastQC. The grey box plots (box: interquartile ranges 25-75 %, whiskers: 10-90 %, and dark bar inside box: median) show the nucleotide-calling Phred quality score across all reads. (A) DK-2011-05-23-9, (B) DK-2010-10-10-3, (C) DK-2003-6-5, (D) DK-2003-7-2, (E) DK-2008-10-5-2, (F) DK-2003-2-3, (G) DK-2004-2-1, (H) DK-2008-10-1-3, (I) DK-2012-01-11-3, (J) DK-1997-19407B, (K) DK-2004-1-7, (L) DK-2011-030311-1. Next generation sequence platforms: A and B: Illumina HiSeq2000, C to L: Ion Torrent PGM™ Sequencer.



Supplementary Fig. 2. Depth of coverage plot. The *x*-axes represent the coverage of every single nucleotide position in respect to the reference sequence and the *y*-axes represent the sequence depth of every single nucleotide. (A) DK-2011-05-23-9, (B) DK-2010-10-10-3, (C) DK-2003-6-5, (D) DK-2003-7-2, (E) DK-2008-10-5-2, (F) DK-2003-2-3, (G) DK-2004-2-1, (H) DK-2008-10-1-3, (I) DK-2012-01-11-3, (J) DK-1997-19407B, (K) DK-2004-1-7, (L) DK-2011-030311-1. Next generation sequence platforms: A and B: Illumina HiSeq2000, C to L: Ion Torrent PGM™ Sequencer.



Manuscript II

Genetic and antigenic characterization of complete genomes of Type 1 Porcine Reproductive and Respiratory Syndrome viruses (PRRSV) isolated in Denmark over a period of 10 years

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(Submitted for publication)

Abstract

Porcine Reproductive and Respiratory Syndrome (PRRS) caused by the PRRS virus (PRRSV) is considered one of the most devastating swine diseases worldwide. PRRS viruses are divided into two major genotypes, Type 1 and Type 2, with pronounced diversity between and within the genotypes. In Denmark more than 50% of the herds are infected with Type 1 and/or Type 2 PRRSV. The main objective of this study was to examine the genetic diversity and drift of Type 1 viruses in a population with limited introduction of new animals and semen. A total of 41 ORF5 and 38 ORF7 nucleotide sequences were obtained from viruses collected from 2003 to February 2013. Phylogenetic analysis of ORF5 nucleotide sequences showed that the Danish isolates formed two major clusters within the subtype 1. The nucleotide identity to the subtype 1 protogenotype Lelystad virus (LV) spanned 84.9-98.8 % for ORF5 and for 90.7-100 % for ORF7. Among the Danish viruses the pairwise nucleotide identities of ORF5 and ORF7 were 81.2-100 % and 89.4-100 %, respectively. Sequencing of the complete genomes, including the 5' and 3'-end nucleotides, of 8 Danish PRRSV Type 1 showed that the genome lengths differed from 14,876 to 15,098 nucleotides and the pairwise nucleotide identity among the Danish viruses was 86.5-97.3 % and the identity to LV was 88.7-97.9 %. The study strongly indicated that there have been at least two independent introductions of Type 1 PRRSV in Denmark and analysis of the full genomes revealed a significant drift in several regions of the virus.

Keywords:

PRRSV, genotype 1/Type 1, complete genome, ORF5, NSP2

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is considered one of the most devastating and economically challenging diseases to the swine industry worldwide (Neumann et al., 2005). The causative agent is the PRRS virus (PRRSV), a small, enveloped single-stranded positive-sense RNA virus belonging to the *Arteriviridae* family, within the order *Nidovirales* (Cavanagh, 1997; Conzelmann et al., 1993). The PRRSV genome is 15-15.5 kb in length with a 5' cap and a 3' polyadenylated tail and it encodes at least 10 open reading frames (ORFs) (Firth et al., 2011; Johnson et al., 2011; Snijder and Meulenberg, 1998; Wu et al., 2001). ORF1a and ORF1b covers the first three quarters of the PRRSV genome and encodes two long non-structural polyproteins, pp1a and pp1ab, where the latter is expressed after a ribosomal frameshift (Snijder and Meulenberg, 1998). pp1a and pp1ab are proteolytically cleaved into at least 12 non-structural proteins (nsps) (Snijder and Meulenberg, 1998; Fang and Snijder, 2010). ORF2-5 encodes the membrane glycoproteins, GP2-GP5, and ORF6 and ORF7 encodes a non-glycosylated membrane protein (M) and the nucleocapsid (N) protein, respectively. Two small genes, ORF2b fully embedded in ORF2 and ORF5a partially overlapping ORF5 encodes the non-glycosylated proteins E and ORF5a protein (Wu et al., 2001; Johnson et al., 2011; Firth et al., 2011). The PRRS symptoms were first described in the late 1980s in pigs in North America and a few years later similar disease symptoms were reported in Europe (Keffaber, 1989; Wensvoort et al., 1991). Genetic nucleotide sequence comparisons of viruses isolated in Europe and North America respectively revealed only 50-60 % identity between viruses; hence PRRSV was divided into two major genotypes, Type 1 and Type 2 (Allende et al., 1999). Genetic diversity is also pronounced within the genotypes, and Type 1 has been divided into at least three subtypes based on the length of ORF7, where the protogenotype 1, the Lelystad virus (LV, Genbank: M96262), belongs to subtype 1 (Stadejek et al., 2002; Stadejek et al., 2006; Stadejek et al., 2008; Forsberg et al., 2002). While PRRSV Type 1, subtype 1 isolates are found in Central and Western Europe and globally, subtypes 2, 3 and the putative subtype 4 have only been found in Eastern European countries east of Poland (Stadejek et al., 2008).

In Denmark, PRRS was first diagnosed in March 1992 in a region with a high-density swine population in the southern part of Jutland close to the border of Northern Germany (Botner et al., 1994). Until 1996, only Type 1 PRRSV circulated in the Danish swine herds, but due to use of Type 2 Ingelvac PRRS MLV attenuated live vaccine that reverted back to high virulence in the field, Type 2 PRRSV was introduced into the Danish pig population (Botner et al., 1997; Madsen et al.,

1998). Today, both Type 1 and Type 2 PRRSV are endemic in Denmark with more than 50% of the herds being infected. Vaccination to control the disease caused by both PRRSV genotypes has since then been used in Denmark. The vaccine Porcilis PRRS (MSD Animal Health) is the only licensed modified live vaccine against Type 1 PRRSV in Denmark. This vaccine was first introduced in Denmark in the beginning of 2001; however, use of this vaccine against Type 1 PRRSV was not consolidated until at the end of the decade. This vaccine is only licensed for use in slaughter pigs, whereas in the rest of Europe it is also licensed for breeding animals. Extensive sequencing of circulating PRRSV isolates during the 1990s revealed that only subtype 1 of the Type 1 PRRSV strains was circulating in Denmark but only few Danish PRRSV isolates have been sequenced since then. The export of Danish pigs to Eastern and central Europe is significant i.e. in 2012 Denmark exported more than 9 million living pigs and more than ¼ of a million breeding animals whereas only few (less than 100) breeding animals were imported to Denmark. Despite this limited import of living pigs the possibility that foreign PRRSV isolates may be introduced into Denmark by contaminated transport carriers or persons cannot be excluded but this will probably be rare events. Nevertheless, the diversity of PRRSV circulating in Denmark is of mutual interest for a range of European pig producing countries. The main objective of this study was to close the gap in knowledge on the genetic diversity of currently circulating PRRS Type 1viruses in Danish pigs and to investigate the genetic drift of the virus in a population with limited possibility of new strains being introduced. Extensive analyses of ORF5 and ORF7 sequences obtained from viruses isolated between 2003 and primo 2013 were examined and compared to sequences obtained from viruses isolated from Danish pig herds in the 1990s. Furthermore, for the first time the complete genome analysis of Danish Type 1 viruses is presented and compared to other Type 1 complete genomes isolated in Europe, North America, and Asia. The results revealed that two viral clusters are circulating, one Lelystad -like and a unique ‘Danish’ cluster.

2. Materials and methods

2.1. Sample material

Lung tissues, serum, and nasal swabs included in this study were obtained from 27 Danish swine herds in the years 2003 to 2013. A number of strains were proliferated in porcine alveolar macrophages (PAM) as previously described (Wensvoort et al., 1991).

2.2. RNA extraction

Total RNA was extracted from serum, nasal swabs, cell culture supernatant, or lung tissue. Lung tissue was prepared as a 5 % homogenate in RLT buffer (QIAGEN) containing 1 % β-mercaptoethanol (Sigma-Aldrich). RNA was extracted from lung homogenate and nasal swabs using RNeasy Minikit (QIAGEN) according to the manufacturer's instructions. Total RNA from serum and cell culture supernatant was purified using QIAamp Viral RNA Mini Kit (QIAGEN). Elution volume for both extractions methods was 60 µl. The RNA was stored at -80°C until use.

2.3. Real time RT-PCR

Purified RNA was initially screened for PRRSV using a modification of the Primer Probe Energy Transfer RT-PCR (PriProET-RT-PCR) assay described by Balka et al. 2009 (Balka et al., 2009).

2.4. cDNA synthesis and PCR amplification

Full-genome length cDNA synthesis was performed by SuperScript® III First-Strand Synthesis System (Invitrogen) following the recommendations by the manufacturer except that the cDNA synthesis step was extended to 90 minutes. A poly(dT) RT-primer was used (5'-CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT $T(T)_{38}$-3' as cDNA primer (Nielsen et al., 2003). PCR amplification of ORF2 to ORF7 was carried out using AccuPrime™ *Taq* DNA Polymerase High Fidelity Kit (Invitrogen). The PCR mixture was prepared as recommended by the supplier except that the amount of AccuPrime™ Taq High Fidelity was increased to 0.5 µl. The PCR cycling was as follows: [94°C for 15 seconds], 45 cycles: [94°C 15 seconds, 55°C for 30 seconds, 68°C for 60 seconds] then finalized with 68°C for 5 minutes and cool down to 4°C. PCR cycling was performed on a T3 Thermocycler (Biometra). PCR primers for amplifying ORF2 to ORF7 were from Diaz, 2006 (Diaz et al., 2006). The PCR products were analyzed by agarose gel electrophoresis using E-gel 2 % agarose gels (Invitrogen) and purified with High Pure PCR Product Purification kit (Roche).

Long range PCR amplification for full genome sequencing was performed using full-genome length cDNA as template with AccuPrime™ Taq High Fidelity kit as described by Kvisgaard et al. 2013.

2.5. Cycle sequencing and next generation sequencing

PCR products of ORF2 to ORF7 were sequenced by cycling sequencing using the Sanger method (Sanger et al., 1977). In total, 30 ORF5 sequences (accession no. KC862504-33) and 27 ORF7 sequences (KC862534-60) were obtained. Three (3) sequences each covering ORF2 to ORF7 (KC862562-64) were assembled from 7 overlapping PCR products. Eight (8) full genome sequences (KC862566-69; KC862571-74, Table 1) were produced from long range PCR amplicons covering the full genome of PRRSV in two or four fragments and sequenced by next generation sequencing technologies. For a detailed description of the procedure and next generation sequencing see Kvisgaard et al. 2013. In short, equimolar concentrations of the PCR amplicons covering the full genome of PRRSV were prepared for sequencing on Roche/454 Genome Sequencer FLX + Titanium (LGC Genomics GmbH, Berlin, Germany), Illumina HiSeq2000 (ARK genomics The Roslin Institute, University of Edinburgh), or Ion Torrent GPM sequencer (DTU Multi-Assay Core (DMAC), Kgs. Lyngby, Denmark).

2.6. Data analyses

The contigs of ORF2 to ORF7 were produced from assembling the raw data obtained by cycle sequencing against the PRRSV Type 1 reference sequence Lelystad (LV) (Genbank: M96262) using the commercial software CLC Main Workbench v. 6.6.2 (CLC BIO, Aarhus, Denmark). Mapping of the reads obtained from the full genome sequencing was performed by the Burrows-Wheeler aligner (BWA) using the Aln algorithm for the Illumina HiSeq2000 data and the bwasw algorithm for Roche 454 FLX and Ion Torrent PGM sequencer data (Kvisgaard et al., 2013). Amino acid sequences were predicted from the nucleotide sequences using CLC Main Workbench v. 6.6.2. Nucleotide and amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log- Expectation). Phylogenetic trees were constructed from Neighbor Joining Algorithm with Bootstrap: 1,000 replicates (CLC Main Workbench v. 6.6.2, CLC BIO, Arhus, Denmark).

The rate of Synonymous (dS) and non-synonymous (dNS) substitutions in each ORF of the 8 full genome sequences were calculated by Synonymous Non-synonymous Analysis Program (SNAP) (http://hcv.lanl.gov/content/sequence/SNAP/SNAP.html) (Körber, 2000). Estimates of amino acid substitutions were determined by the method of dS-dNS rates: dS-dNS > 0, dS-dNS < 0, and dS-dNS = 0 representing tendencies for synonymous and non-synonymous variations, and neutral mutations, respectively.

Potential N-glycosylation sites were determined for GP2, GP3, GP4, and GP5 using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). A potential glycosylation site was accepted when the potential score was above the minimum threshold of 0.5 and the agreement between nine neural networks ('the jury') was 9/9 with minimum ++ (- = potential < 0.5 (threshold), + = potential > 0.5, ++ = potential > 0.5 and Jury agreement (9/9), +++ = potential > 0.75 and Jury agreement) (http://www.cbs.dtu.dk/services/NetNGlyc/output.php).

3. Results

3.1. Genetic diversity of ORF5 and ORF7

A total of 41 ORF5 and 38 ORF7 nucleotide sequence data were obtained from 44 viruses collected from 2003 to February 2013. The nucleotide lengths of the ORF5 and ORF7 sequences were 606 and 387 nucleotides, respectively. A single virus (DK-2012-10-01-27/KC862521) had an ORF5 nucleotide length of 603. The nucleotide identity to the subgroup 1 genotype Lelystad virus (LV) (M96262) spanned for ORF5 84.9-98.8 % and for ORF7 90.7-100 % and among the Danish viruses the pairwise nucleotide identities of ORF5 and ORF7 were 81.2-100 % and 89.4-100 %, respectively.

3.2. Phylogenetic analysis

The nucleotide sequences from this study were aligned with previously sequenced Danish viruses (Forsberg et al., 2002) obtained from Genbank and representative viruses from Europe, Asia, and North America. Phylogenetic trees based on the nucleotide sequences of ORF5 and ORF7 are shown in Fig. 1. The phylogenetic analysis of ORF5 nucleotide sequences showed that the Danish viruses formed two major clusters. The majority of the Danish viruses isolated in the period 2008-2013 clustered together with the LV and the Porcilis vaccine viruses with a pairwise nucleotide identity of 95.2-98.8 % and 95.7-99.8 %, respectively (I, Fig. 1A). The other Danish cluster (II, Fig. 1A) constituted Danish viruses collected prior to 2008 but also a few viruses from 2011-2012 with nucleotide identity to LV of 84.9-93.6 %. One virus with a 3 nucleotide deletion in ORF5 belonged to this cluster. Interestingly, this cluster contained only Danish viruses and a single virus from China isolated in 2009. The Danish ORF7 sequences also clustered into 2 major clusters (I-II, Fig. 1B), with a pairwise identity to LV of 95.6-100 % and 90.7-96.1 %, respectively.

3.3. Amino acid analysis of GP5 and N proteins

Examination of the deduced amino acid sequences from ORF5 showed that 105 of the 201 positions in GP5 were conserved in all of the 58 Danish isolates (52.0 % conserved sites). Forty-nine positions varied in more than one isolate, and 19 positions varied in more than 10 isolates. At position aa122 and aa172, the amino acid residues leucine/phenylalanine and glycine/aspartic acid, respectively were equally distributed, hence a consensus amino acid at these two positions was not possible to determine. Three potential N-glycosylations sites, N37, N46, and N53, were conserved in 90 %, 100 %, and 100 % of the viruses, respectively. The glycosylation site N37 was not present in 6 Danish viruses isolated between 1992 and 2007 which is in accordance with this glycosylation site not being present in the Type 1 reference strain LV. The cysteine residue thought to be involved in heterodimer formation with the M protein at position 50 (Verheije et al., 2002) was conserved in all the Danish sequences. The previously predicted (Plagemann, 2004; Oleksiewicz et al., 2002) major neutralizing epitope (aa38-54) and epitope site ES13 (aa178-200) were conserved in the consensus sequence of Danish GP5 sequences. One of the highly variable positions (aa122) was located in a putative T-cell epitope (Diaz et al., 2009) (Fig. 2A). A histogram illustrating the variability among the 58 Danish viruses is shown in Fig. 2B. The major neutralizing epitope located at the amino acid position aa38-54 was highly conserved in all the Danish viruses, which was also the case for the ES13 epitope (Oleksiewicz et al., 2002).

The examination of the deduced nucleocapsid protein revealed less variation between the Danish viruses compared to the GP5 protein (Fig. 3). Eighty-nine out of 128 amino acid positions in the N protein were conserved (69.5 % conserved sites). Twenty positions varied in more than one isolates, and only 4 positions varied in more than 10 isolates. The two cysteine residues at position 27 and 76 thought to be crucial for homodimer formation of the N protein were conserved throughout all the Danish isolates as was the putative linear B-cell epitope at position 80-88 described previously (An et al., 2005). The four predicted T-cell epitopes (Diaz et al., 2009) where highly conserved, with a minor variation at position aa64 where 8 isolates harbored an amino acid substitution. The most variable position in the N protein among the Danish viruses was at aa13, with 26 viruses harboring substitution (Fig. 3).

3.4. Genetic analysis of complete genomes

Complete genomes, including the very 5’ and 3’-end nucleotides, of 8 Danish PRRSV Type 1 isolated in the years 1992 to 2012 were determined and the sequences analyzed and compared to

published reference sequences. The genome lengths differed from 14,876 to 15,098 nucleotides (excluding the Poly(A) tail) and the pairwise nucleotide identity among the Danish viruses was 86.5-97.3 % and the isolates were 88.7-97.9 % identical to the LV strain (Table 1). The analysis of synonymous - non-synonymous (dS-dNS) mutations among the 8 Danish genomes showed a substantial higher amino acid substitution rate in ORF1a than in ORF1b (Fig. 4). The dNS mutations in the genes encoding the structural proteins were scattered throughout the genomes, with the highest rates in ORF3 and ORF5 (Fig. 4).

A global phylogenetic analysis of PRRSV Type 1 ORFs 1-7 (Fig.5) showed that the Danish viruses divided into two clusters as seen for the ORF5 and ORF7 phylogenetic analyses (Fig. 1). Five of the Danish isolates clustered with LV, Porcilis PRRS, and two viruses isolated in Germany 1992 and Thailand 2006. The three remaining Danish viruses grouped separately with a Chinese isolate from 2009.

3.5. The non-structural proteins

In Table 2 the pairwise nucleotide and amino acid identity of the non-structural proteins (nsp) of the Danish viruses are listed. The lowest average amino acid variation was found in nsp6, nsp11, nsp8, and nsp12, respectively. The highest variation was found in nsp1α/β and nsp2 with the amino acid identity range of 86.2-97.9 % and 77.0-97.7 % respectively. The predicted epitope site ES1 in nsp1α/β at position aa236-252 showed high variation with a substitution rate of 41 % among the Danish viruses. A comparison of the predicted amino acid sequences of the nsp2 of the Danish isolates with 19 previous published sequences of Type 1 PRRSV viruses is shown in Fig. 6. The lengths of nsp2 for the Danish viruses varied from 1004 to 1078 amino acids. Interestingly, DK-2008-10-5-2 and DK-2012-01-05-2 had a 74 amino acid deletion also seen in the Porcilis PRRS strain (Darwich et al., 2011). These deletions spanned the predicted ES3 (Oleksiewicz et al., 2001) and T-cell epitope (Fang et al., 2004). Furthermore, a three amino acid deletion was discovered in DK-2003-6-5 located in ES7. The B cell epitope sites ES3, ES4, and ES7 together with the T-cell epitope were all located in highly variable regions, with substitution rates of 66%, 74 %, 74 % and 87%, respectively.

3.6. The structural proteins

Comparisons of the deduced amino acid sequences of the structural proteins of the Danish isolates revealed that the highest variation among the 8 Danish viruses was in GP3 and GP4 with an

average similarity of 88.3 ± 4.3 and 90.4 ± 3.6, respectively (Table 2). Both GP3 and GP4 induce neutralizing antibodies (Oleksiewicz et al., 2001; Oleksiewicz et al., 2002) and encode the B cell epitopes ES11 and ES12 at position aa60-87 and aa59-70 relative to GP3 and GP4 of LV, respectively. The amino acid sequences of GP3 of the eight isolates were aligned with the GP3 of additional 27 Danish viruses isolated in 2010 and in the 1990s (Forsberg et al., 2001) (Fig 7A). The ES11 showed a substitution rate of 46 % among the 35 Danish viruses (Fig. 7A). In comparison to the LV strain, DK-2003-7-2 had a deletion in GP3 of 8 amino acids at the position aa240-247 (Fig. 7B). This deletion has previously been detected in three Asian viruses (GU047344, GU047345, and EU076704) and eight Danish viruses isolated in the 1990s also had 1 to 8 amino acids deletions in this area (Fig. 7B). This region has previously been found to contain a B-cell epitope (243RKASLSTS250) (Oleksiewicz et al., 2000) but this antigenic motif is not present in any of the viruses examined in the present study (Fig. 7B). The ORF3 and the ORF4 coding regions overlap and hence any deletion in this shared coding region is likely to affect both protein products, which also was the case for DK-2003-7-2 which with an 8 amino acid deletion in GP4 at the position aa58-65 in LV (Fig. 8). The ES12 of GP4 is located in a region prone for deletions and besides that, it is also a highly variable region with a 100 % substitution rate among the Danish viruses (Fig. 8).

Examination of potential N-glycosylation sites revealed 2 putative N-glycosylation sites in GP2, 7 putative N-glycosylation sites in GP3 and 4 putative N-glycosylation sites in GP4 (Table 3). The result of nine neural networks predicted that both putative N-glycosylation sites in GP2 (N173 and N179) were glycosylated, although one virus (DK-2010-10-12-1) did not score an unanimous vote from the jury network. For GP3 only N27, N50, N130, and N194 were potentially N-glycosylated. For N27, three Danish viruses were not predicted to harbor N-glycosylation at this site. For GP4 only N88 and N124 were predicted to be N-glycosylated (Table 3).

Cysteine residues important for protein folding and function were 100 % conserved in all the Danish viruses examined.

4. Discussion

There has been a gap in knowledge regarding the diversity of PRRSV Type 1 circulating in Denmark since the latest available information was obtained on viruses isolated in the late 1990s.

Most work on the diversity of European PRRS Type 1 viruses has focused on the structural proteins encoding genes, mainly ORF5, and ORF7 (Oleksiewicz et al., 2000; Forsberg et al., 2002). Here we report an extensive analysis of ORF5 and ORF7 of 48 viruses and full genome sequences

of 8 Type 1 PRRS viruses isolated over almost two decades which to our knowledge make this study the most extensive Type 1 study published globally. The phylogenetic analyses of ORF5 and ORF7 revealed that the viruses isolated in Danish herds between 2003 and primo 2013 were highly diverse and clustered into two distinct phylogenetic groups. Most of the viruses grouped with the Type 1 reference strain LV which is highly identical to the Porcilis vaccine strain, however a few recent viruses clustered into a phylogenetic group that contained only the Danish viruses and these isolates were genetically different from the LV strain. Interestingly, only viruses isolated after the Porcilis vaccine were introduced in Denmark grouped in the LV cluster which may indicate that the Porcilis strain may be circulating in Danish herds. That the Danish PRRSV Type 1 viruses divide into two phylogenetic groups was also shown in the study by Forsberg et. al, 2002 (Forsberg et al., 2002). That study also indicated the existence of two Danish clusters in that two viruses isolated in 1993 and 1995 grouped with LV and were therefore called 'LV-like, although they only shared 93.6 % pairwise identity to LV. LV was the first European PRRSV Type 1 isolated, and the sequence of this virus has been used as reference sequence in genetic comparisons, however based on the grouping in the phylogenetic tree not all the Type 1 viruses in Denmark may be progenies of LV (Shi et al., 2010). Accordingly, one of the first sequenced Danish PRRS viruses, DK111-92 (AJ223078) isolated in 1992, showed a pairwise nucleotide identity to LV of only 93.1 % in ORF5. These analyses of early Danish PRRSV viruses suggested that more than one PRRSV Type 1 strain circulated in Europe and that Type 1 PRRSV was introduced into Denmark upon at least two different occasions. The presence of diverse Type 1 isolates in Europe at such an early time point indicates that Type 1 viruses have undergone a pronounced genetic drift or that the virus has been circulating a long time before clinical signs became evident. These findings are in agreement with the observation that Type 1 PRRSV isolated in Italy and United Kingdom clustered in two groups, one LV like and one Italian-/British-like (Forsberg et al., 2002; Frossard et al., 2013; Shi et al., 2010). Despite the high level of diversity among the Danish ORF5 nucleotide sequences, the deduced amino acid sequence of GP5, showed high level of conservation at the positions of putative N-glycosylation sites, cysteine residues important for heterodimer formation with M protein, and also the putative neutralizing epitope was well conserved throughout all the Danish viruses.

In conclusion, the analysis of the ORF5 and ORF7 sequences indicated the presence of two circulating virus clusters, one dominating vaccine/LV like and one resembling an early introduced strain. Importantly, the analysis also confirmed that all the Danish isolates fall within genotype 1, and are distinct from the recently described Eastern European subgroups (Stadejek et al., 2008).

The sequencing of the complete genomes of Danish PRRS field viruses confirmed that there is a high overall diversity among Type 1 viruses in Europe. Thus, the pairwise nucleotide variation over the complete genome was 2.7-5.8 %. Even among the most recently isolated viruses (2010-2012). The variation within the viral genome showed to be unequally distributed as NSP1α/β, NSP2, ORF3, and ORF4 showed the highest level of variation with 9.2 %, 12.4 %, 9.2 %, and 9.7 % average variation, respectively. The genomic regions with the lowest variation were ORF6 and ORF7 with 5.1 % and 4.6 % average variation, respectively. ORF1b showed a relatively high average nucleotide variation of 7.7 %, however, most of the variations were synonymous, and consequently the average amino acid variation was only 3.1 %. This level of amino acid conservation correlates well with the fact that this part of the viral genome encodes the key enzymes; RNA dependent RNA polymerase (nsp9) and helicase (nsp10) important for the RNA replication and unwinding of RNA duplexes, respectively (Fang and Snijder, 2010). The overall diversity of the Danish viruses fits very well with the global picture of Type 1 PRRSV diversity recently published by Darwich et.al (Darwich et al., 2011).

The nsp2 protein is the largest of the mature PRRSV proteins and has been shown to be critical in proteolytic processing of proteins active in virus replication and modulation of the immune response (Fang and Snijder, 2010; Han et al., 2009). The N-terminal of nsp2 possesses a putative cysteine protease domain of about 100 amino acids (aa36-aa140, Fig. 6). This cysteine domain is likely to be required for nsp2/3 proteolysis (Han et al., 2007). Nsp2 is the viral protein containing most B-cell epitopes (Oleksiewicz et al., 2001) and the antibody response to nsp2 is greater than towards any other PRRSV protein, however the antibodies raised against this protein is non-neutralizing (Johnson et al., 2007; Brown et al., 2009). One epitope, ES2, located in the putative cysteine protease domain, showed high level of conservation among the Danish viruses and globally, which agrees with the earlier findings that this domain is important for viral survival, as deletion of the domain is lethal to the virus (Han et al., 2007).The length of the nsp2 protein has been reported to be highly variable (Fang et al., 2004; Han et al., 2006) which also was the case for the Danish viruses in this study (1004-1078aa). DK-2008-10-5-2 and DK-2012-01-05-2 contained a 74 amino acid deletion at the exact same position as the Porcilis PRRS vaccine; however the pairwise nucleotide identity of the two Danish viruses and the vaccine strain was only 95.8 % and 97.4 % indicating that this deletion cannot be used as a marker for the vaccine strain. Interestingly, this region span the epitope sites ES3 and the putative T-cell epitope and may therefore be prone to changes which are supported by the findings that other PRRSV Type 1viruses also harbored

deletions in the same region (Fig. 6). One Danish virus harbored a 3 amino acid deletion in ES7 which has not previously been reported. The significance of these epitope sites in nsp2 was confirmed by a study by Chen et. al (Chen et al., 2010) showing that deletion of ES3 actually increased the cytolytic activity and showed more vigorous growth kinetics in vitro and produced a higher vial peak load in pigs. The opposite was seen for deletions of ES4 and ES7. The impact of the ES3deletion in nsp2 of the vaccine strains should be investigated more thoroughly since it may suggest that the Porcilis PRRS vaccine strain has the ability to replicate more efficiently.

Of the structural proteins, the highest degree of variation was seen in GP3 and GP4 (Table 2). Especially the areas predicted to contain epitopes underwent higher mutation rates and harbored more deletions than those motifs crucial for protein folding and functions, such as cysteine and glycosylation sites. From the deduced amino acid sequences of GP2, GP3, and GP4, 2, 7 and 4 putative N-glycosylation motifs were present in all the Danish sequences, thus only four sites in GP3 and two sites in GP4 were predicted to be N-glycosylated by a neural network. Both putative N-glycosylation sites predicted for GP2were conserved in all the Danish viruses examined, however one virus failed the neural network jury prediction at N173. The finding that not all putative N-glycosylations sites were predicted to be glycosylated even though the N-glycosylation motif (N-X-S/T-X, X ≠ P) was fulfilled can be explained by protein folding leading to steric hindrance for the glycans or simply because glycosylations at these positions is of no advantage for the virus survival/function. The results can also be due to misinterpretation of the neural network in regards to the parameters to be fulfilled for accepting an N-glycosylation site. Accordingly, a previous study of the role of N-glycosylation of GP2 showed that lack of glycosylation at either one or both sites did not affect the formation or infectivity of virions (Wissink et al., 2004). The role of N-glycosylation of GP3 and GP4 of Type 1 PRRSV has not yet been examined in vitro, but such studies have been performed for Type 2 PRRSV (Das et al., 2011). They showed that N-glycosylation of N42, N50, N131 in GP3 (corresponds to N42, N50, N130 in GP3 PRRSV Type 1) was required for infectious virus production, where as any three of the four putative N-glycosylation sites (N37, N84, N120, and N130 in GP4 PRRSV Type 2) were necessary for recovery of infectious virus. From the analysis of N-glycosylation sites in GP4 in this study, only N88 and N124 (equals N84 and N120 in Type 2 PRRSV) were predicted to be N-glycosylated.

In conclusion, this study presents for the first time the examination of complete genomes obtained from PRRS Type 1viruses isolated in a country with very limited import of living animals and semen. Only 18 complete genomes (or nearly complete) of PRRSV genotype 1 sequences were

as of date available for comparison in public databases, where 10 of the genomes were obtained from viruses isolated in Europe in the years 1991 to 2007 (Darwich et al., 2011; Van Doorsselaere et al., 2011; Van Doorsselaere et al., 2012; Meulenberg et al., 1993). Thus, the present work adds valuable data on the PRRSV Type 1 viruses circulating in Europe. Characterization of the genetic and antigenic diversity of PRRSV is mandatory for the development and maintenance of diagnostic tools and for development of new vaccine entities.

5. Acknowledgement

Grateful thanks to Dr. Laila Darwich for kindly providing the genomic sequence of Porcilis PRRS. The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 245141 (New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia (PoRRSCon) coordinated by Prof. H. Nauwynck) and the COST Action FA902: Understanding and combating porcine reproductive and respiratory syndrome in Europe (EuroPRRS.net).

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Table 1. Overview of the 8 Danish Type 1 PRRSV complete genomes

Virus	Accession no.	Collection year	Genome length[a]	Nt identity[b] (%)
DK-1992-PRRS-111_92	KC862566	1992	15,098	92.0
DK-2003-6-5	KC862571	2003	15,089	88.9
DK-2003-7-2	KC862572	2003	15,074	88.7
DK-2008-10-5-2	KC862573	2008	14,876	94.8
DK-2010-10-10-3	KC862568	2010	15,098	97.5
DK-2011-05-11-14	KC862567	2011	15,098	97.9
DK-2011-05-23-9	KC862569	2011	15,098	97.2
DK-2012-01-05-2	KC862574	2012	14,876	96.0

[a]Excluding the Poly(A) tail; [b]Pairwise nucleotide identity to Lelystad (M96262)

Table 2. Pairwise nucleotide and amino acid identity range among the 8 Danish PRRSV Type 1 field viruses and the range of identity to LV. The predicted pp1a and pp1ab protein cleavage sites are from Fang and Snijder, 2010.

ORFs	Length (nt)	% identity range(nt)	Avg. nt identity % (mean ± sd)	% identity rangeto LV (nt)	Proteins	Length (aa)	% identity range(aa)	Avg. aa identity % (mean ± sd)	% identity range to LV (aa)
ORF1a	6969, 7182, 7191	83.3-98.0	90.2 ± 4.5	86.8-97.9	**pp1a**	2276, 2347, 2350	84.8-97.9	90.5 ± 3.7	87.9-98.1
NSP1α/β	1155	84.9-97.4	90.8 ± 4.3	86.7-98.3	**nsp1α/β**	385	86.2-97.9	91.3 ± 3.3	88.1-98.4
NSP2	3012, 3225, 3234	79.1-97.8	87.6 ± 5.1	85.3-98.0	**nsp2**	1004, 1075, 1078	77.0-97.7	86.1 ± 6.1	82.4-97.7
NSP3	690	87.1-99.3	91.8 ± 3.9	87.7-99.4	**nsp3**	230	93.5-99.6	95.8 ± 1.9	94.8-100.0
NSP4	609	85.4-100.0	91.9 ± 4.7	86.5-99.3	**nsp4**	203	91.1-100.0	95.7 ± 2.6	92.6-99.5
NSP5	510	85.7-99.0	92.3 ± 4.5	87.7-99.8	**nsp5**	170	89.4-100.0	94.7 ± 3.1	91.2-100.0
NSP6	48	85.4-100.0	92.3 ± 5.0	85.4-100.0	**nsp6**	16	100.0	100.0	100.0
NSP7α/β	807	87.5-98.3	92.2 ± 3.4	89.5-98.6	**nsp7α/β**	269	91.8-98.88	95.5 ± 2.2	92.9-98.9
NSP8	135	88.4-99.3	93.9 ± 3.0	89.9-99.3	**nsp8**	45	95.6-100.0	97.9 ± 2.0	95.6-100.0
ORF1b	4509[a]	88.1-97.4	92.3 ± 3.2	89.1-98.2	**pp1b**	1502	94.9-99.2	96.9 ± 1.5	95.4-99.1
NSP9	2052	88.8-98.0	92.8 ± 3.1	89.7-98.4	**nsp9**	684	95.3-99.6	97.5 ± 1.4	95.3-99.6
NSP10	1326	86.6-97.2	91.8 ± 3.9	87.8-98.1	**nsp10**	442	92.1-99.1	95.6 ± 2.2	93.4-99.1
NSP11	672	87.8-97.6	92.1 ± 3.2	89.3-97.9	**nsp11**	224	96.9-99.6	98.3 ± 0.8	97.3-99.6
NSP12	456	87.4-96.7	92.1 ± 2.8	89.8-98.3	**nsp12**	152	92.8-99.3	96.1 ± 2.0	93.4-100.0
ORF2	750	89.9-97.3	93.3 ± 2.3	91.1-98.0	**GP2**	249	91.6-97.6	94.0 ± 1.8	93.2-98.4
ORF2b	213	93.4-99.1	96.1 ± 1.3	94.8-99.1	**E**	70	97.1-100.0	96.1 ± 1.3	97.1-98.6
ORF3	798, 774	85.8-96.7	90.8 ± 3.4	87.7-97-7	**GP3**	265, 257	81.1-94.7	88.3 ± 4.3	82.6-96.9
ORF4	528,552	84.2-96.6	90.3 ± 4.2	85.9-96.7	**GP4**	175, 183	84.7-97.3	90.4 ± 3.6	87.4-97.3
ORF5	606	88.5-98.4	93.0 ± 3.1	89.8-98.0	**GP5**	201	89.6-99.0	92.9 ± 2.5	92.0-97.5
ORF5a	132	95.5-98.5	96.4 ± 0.9	97.0-99.2	**ORF5a protein**	43	90.7-100.0	94.8 ± 1.9	93.0-100.0
ORF6	522	91.8-98.5	94.9 ± 1.9	93.5-99.4	**M**	173	93.6-99.4	96.2 ± 1.6	95.4-99.4
ORF7	387	92.5-99.2	95.4 ± 1.9	94.1-99.7	**N**	128	93.8-99.2	96.5 ± 1.5	96.9-100.0

[a]Length calculated from NSP9 coding region in ORF1a minus the ribosomal frameshift

Table 3. Putative N-glycosylation sites in GP2, GP3, and GP4 of the Danish Type 1 PRRSV

Virus	GP2		GP3							GP4			
	N173	**N179**	**N27**	**N42**	**N50**	**N130**	**N151**	**N159**	**N194**	**N37**	**N88**	**N124**	**N134**
DK-1992-PRRS-111_92	9/9 ++	9/9 ++	8/9 +	5/9 +	9/9 ++	9/9 +++	8/9 +	6/9 +	9/9 ++	7/9 +	9/9 ++	9/9 ++	8/9 +
DK-2003-6-5	9/9 ++	9/9 ++	7/9 +	5/9 +	9/9 ++	9/9 +++	8/9 +	7/9 +	9/9 ++	8/9 +	9/9 ++	9/9 ++	8/9 +
DK-2007-7-2	9/9 ++	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	8/9 +	9/9 ++	7/9 +	9/9 ++	9/9 ++	8/9 +
DK-2008-10-5-2	9/9 ++	9/9 ++	8/9 +	5/9 +	9/9 ++	9/9 +++	8/9 +	8/9 +	9/9 ++	8/9 +	9/9 ++	9/9 ++	7/9 +
DK-2010-10-10-3	9/9 ++	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	7/9 -	9/9 ++	7/9 +	9/9 ++	9/9 ++	8/9 +
DK-2011-05-11-14	9/9 ++	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	7/9 -	9/9 ++	8/9 +	9/9 ++	9/9 ++	8/9 +
DK-2011-05-23-9	9/9 ++	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	7/9 -	9/9 ++	7/9 +	9/9 ++	9/9 ++	8/9 +
DK-2012-01-05-2	9/9 ++	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	4/9 +	9/9 ++	7/9 +	9/9 ++	9/9 ++	8/9 +
DK-2010-30-11-11	9/9 ++	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	4/9 +	9/9 ++	7/9 +	9/9 ++	9/9 ++	8/9 +
DK-2010-30-11-14	9/9 ++	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	4/9 +	9/9 ++	7/9 +	9/9 ++	9/9 ++	8/9 +
DK-2010-10-12-1	8/9 +	9/9 ++	9/9 ++	5/9 +	9/9 ++	9/9 +++	8/9 +	7/9 -	9/9 ++	8/9 +	9/9 ++	9/9 ++	8/9 +
	91 %	**100 %**	**73 %**	**-**	**100 %**	**100 %**	**-**	**-**	**100 %**	**-**	**100 %**	**100 %**	**-**

Fig. 1. Phylogenetic analyses of PRRSV Type 1 ORF5 (A) and ORF7 (B) nucleotide sequences. Alignment of the sequences was performed with the MUSCLE algorithm, and phylogenetic analyses (neighbor-joining method with bootstrap 1.000) were conducted using CLC Main Workbench v. 6.6.2 (CLC BIO, Arhus, Denmark). The ORF5 and ORF7nuclotide sequences of protogenotype for PRRSV Type 2 VR2332 were used as outgroups ('set root above node'). The scale bar represents 4 nucleotide changes per hundred. All the Danish isolates are marked in red. The two major groups, I and II, formed by the Danish isolates are identified by black bars.

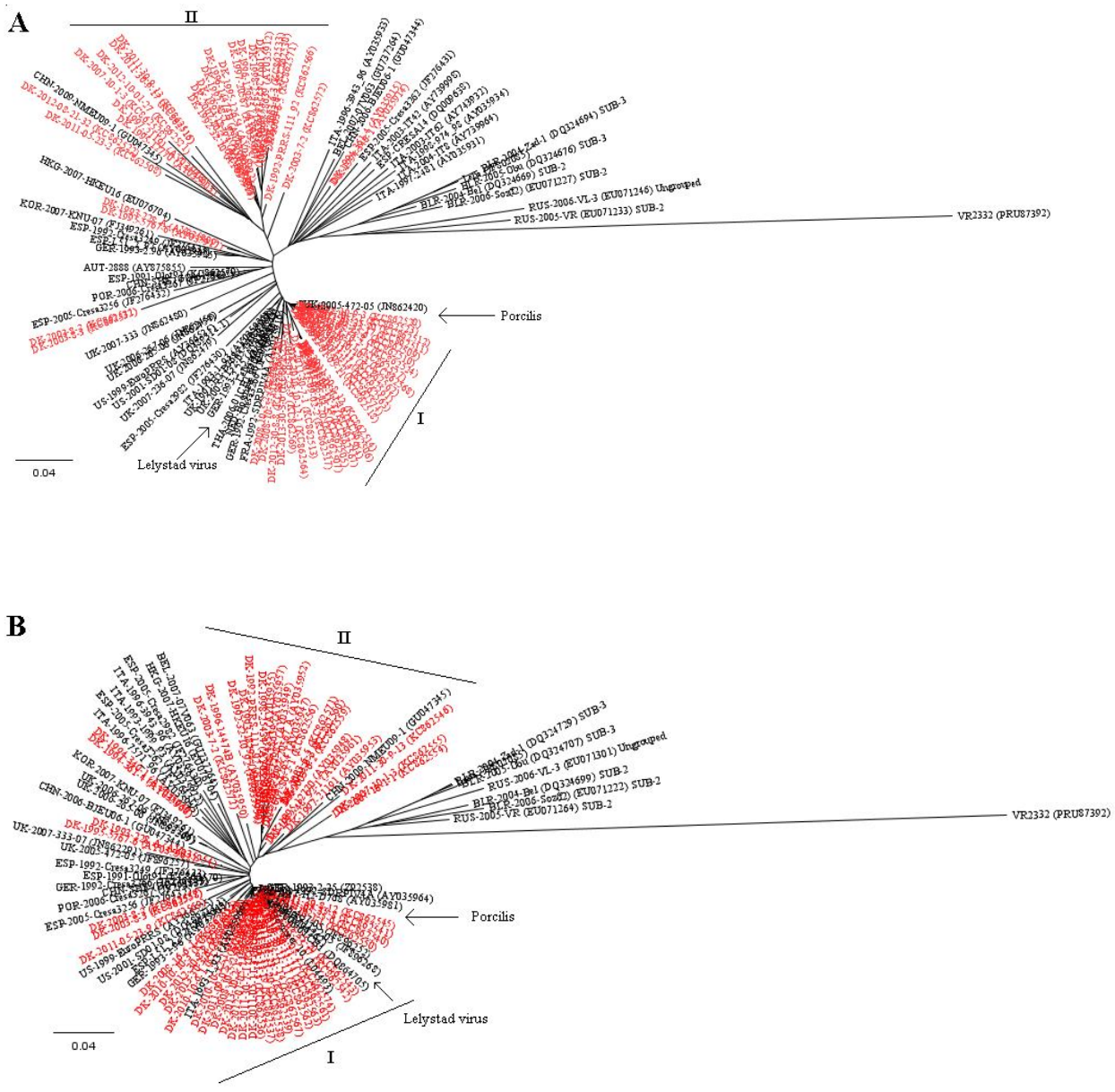


Fig. 2. (A) Consensus amino acid sequence obtained from an alignment of 58 Danish GP5 sequences. Epitope sites are highlighted with black boxes and the three putative N-glycosylation sites are marked with an asterix (*)(NetNGlyc 1.0). (B) A histogram showing the number of substitutions found at each amino acid position in the GP5 of 58 Danish isolates. 'Empty' positions represent conserved amino acids. The three potential N-glycosylation sites at aa37, aa46, aa53 are marked with *. At the amino acid positions aa122 and aa172, the amino acid residues leucine/phenylalanine and glycine/aspartic acid, respectively were equally distributed, hence a consensus amino acid at these two positions was not possible to determine.

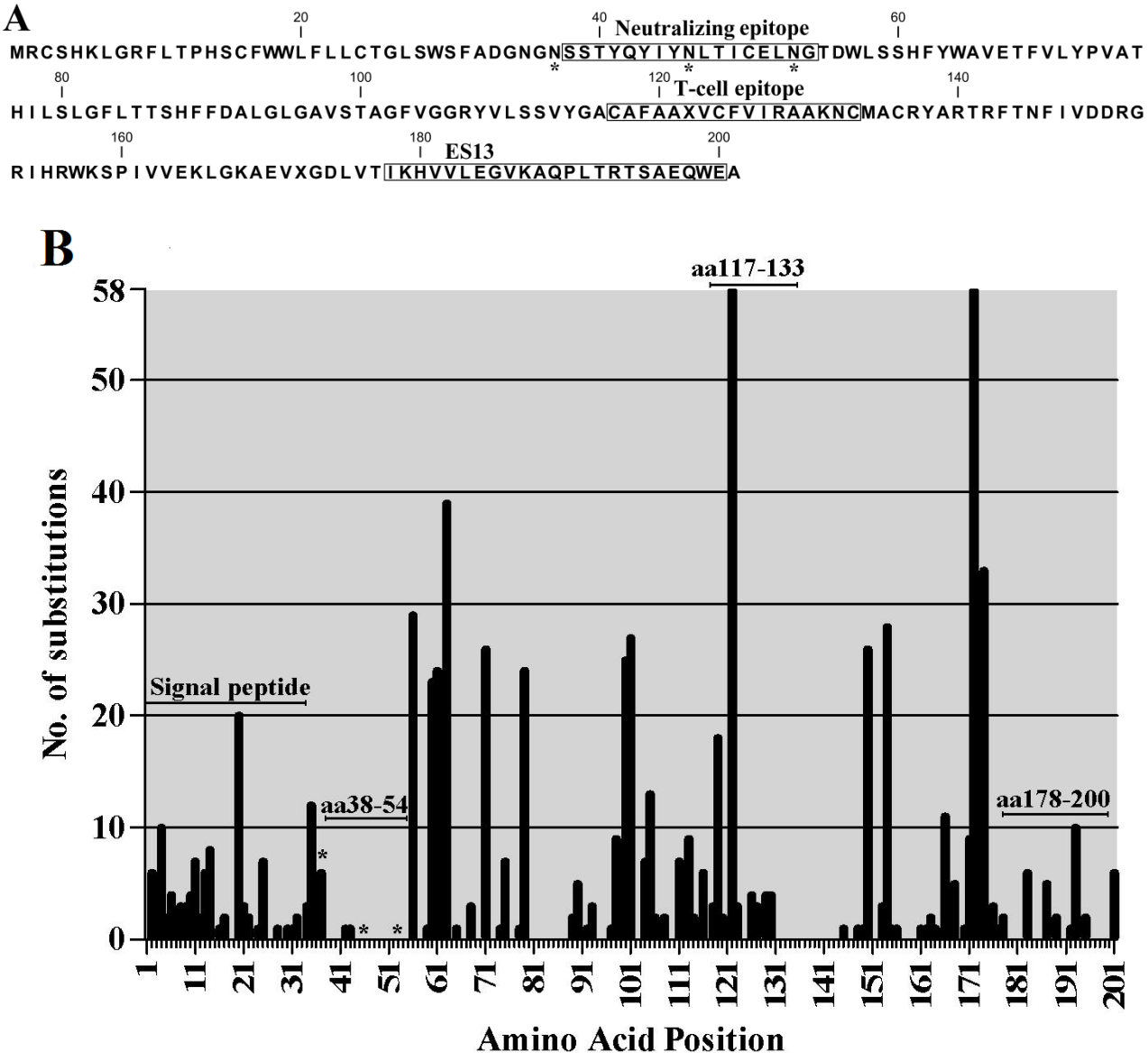


Fig 3. (A) Consensus amino acid sequence obtained from the alignment of 55 Danish PRRSV N protein sequences. Epitope sites are highlighted with black boxes. (B) A histogram showing the number of substitutions found at each amino acid position in N protein of 55 Danish viruses. 'Empty' positions represent conserved amino acids.

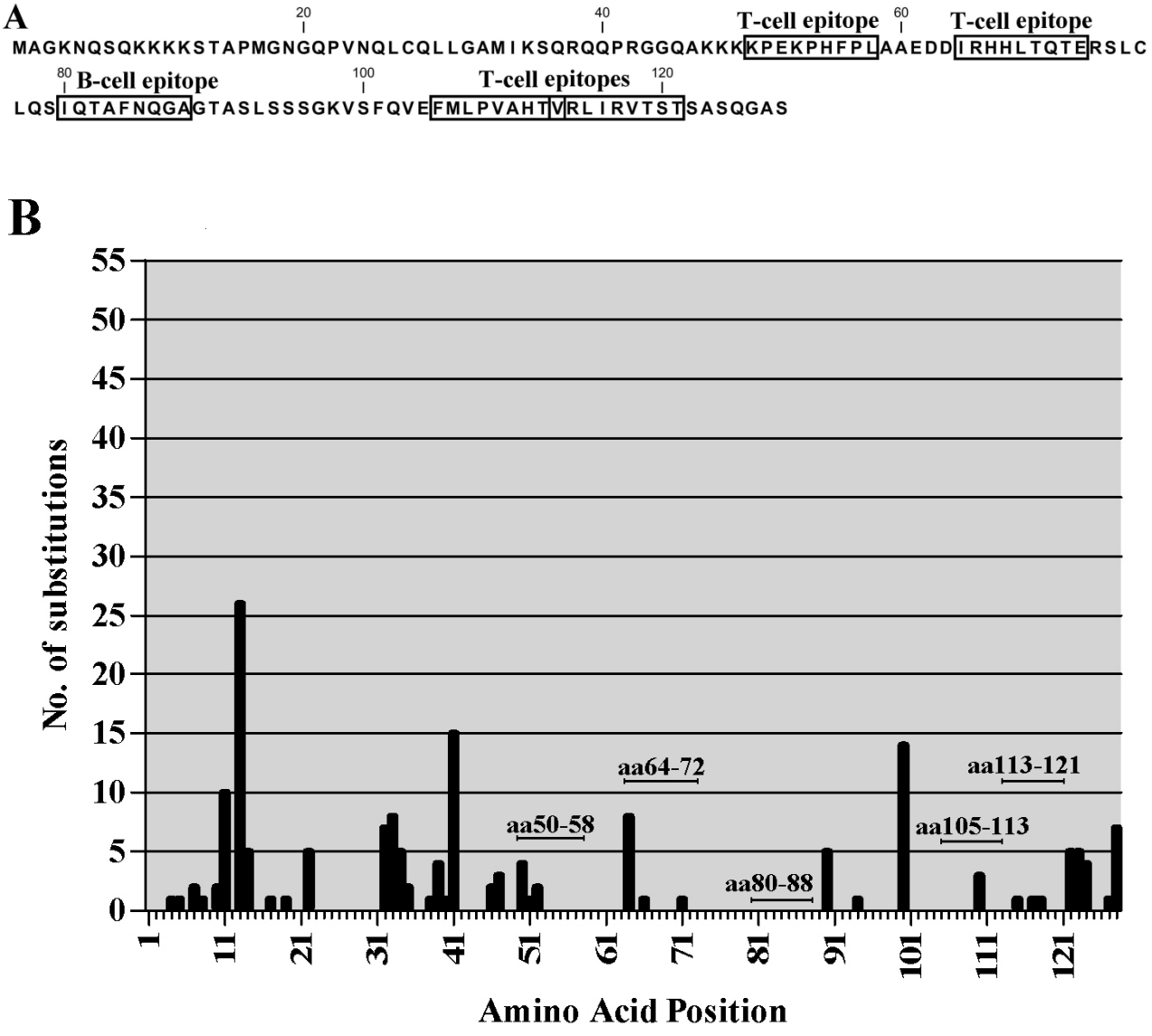


Fig. 4. Topology of synonymous (dS) and non-synonymous (dNS) substitutions in 8 Danish Type 1 viruses. Estimates of amino acid substitutions were represented by the method of dS-dNS rates: dS-dNS > 0, dS-dNS < 0, and dS-dNS = 0 representing tendencies for synonymous and non-synonymous variations, and neutral mutations, respectively. The numbering on the *x*-axis represents selected amino acid position in polyprotein 1a and polyprotein 1ab. aa386, aa1464, and aa3037 represent the putative cleaving sites between nsp1α/β and nsp2, nsp2 and nsp3, and nsp9 and nsp10, respectively in regards to the pp1ab sequence of LV.

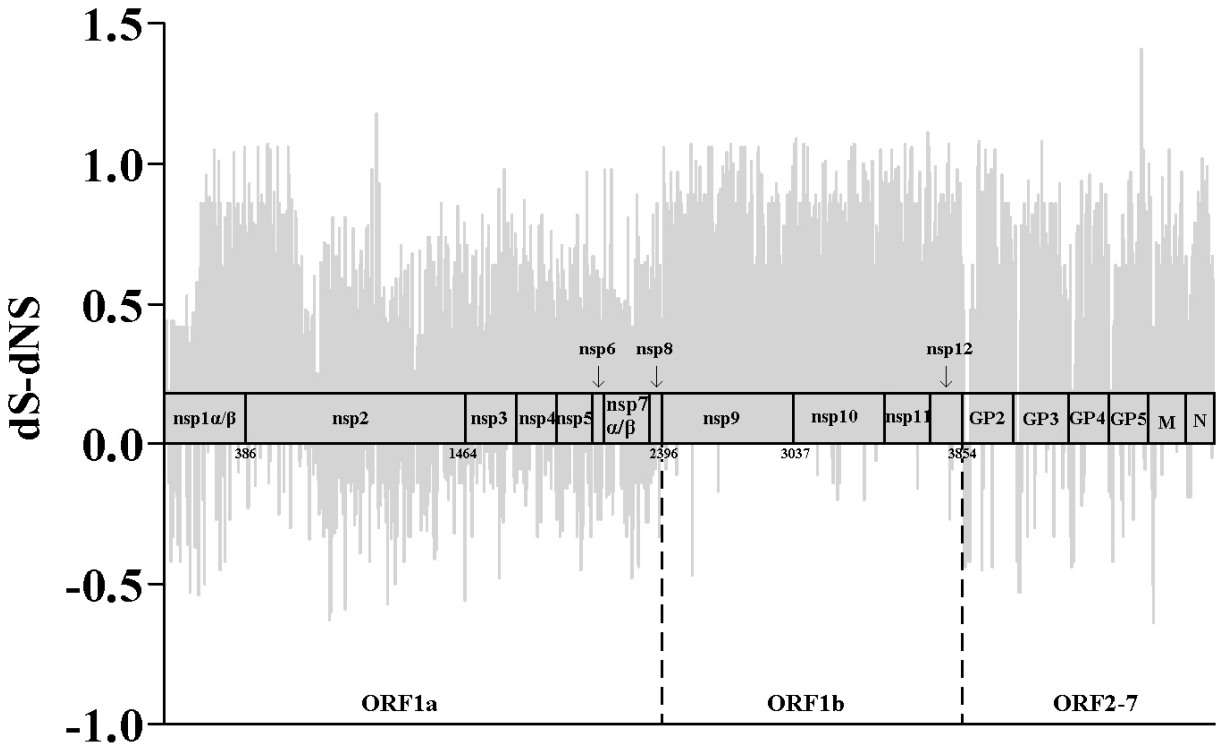


Fig. 5. Phylogenetic analysis of PRRSV Type 1 ORF1-7 nucleotide sequences. Bootstrap values are shown on branches. The scale bar represents 18 nucleotide changes per hundred. BEL = Belgium, CHN = China, DK = Denmark, ESP = Spain, GER = Germany, HKG = Hong Kong, KOR = South Korea, POR = Portugal, THA = Thailand.

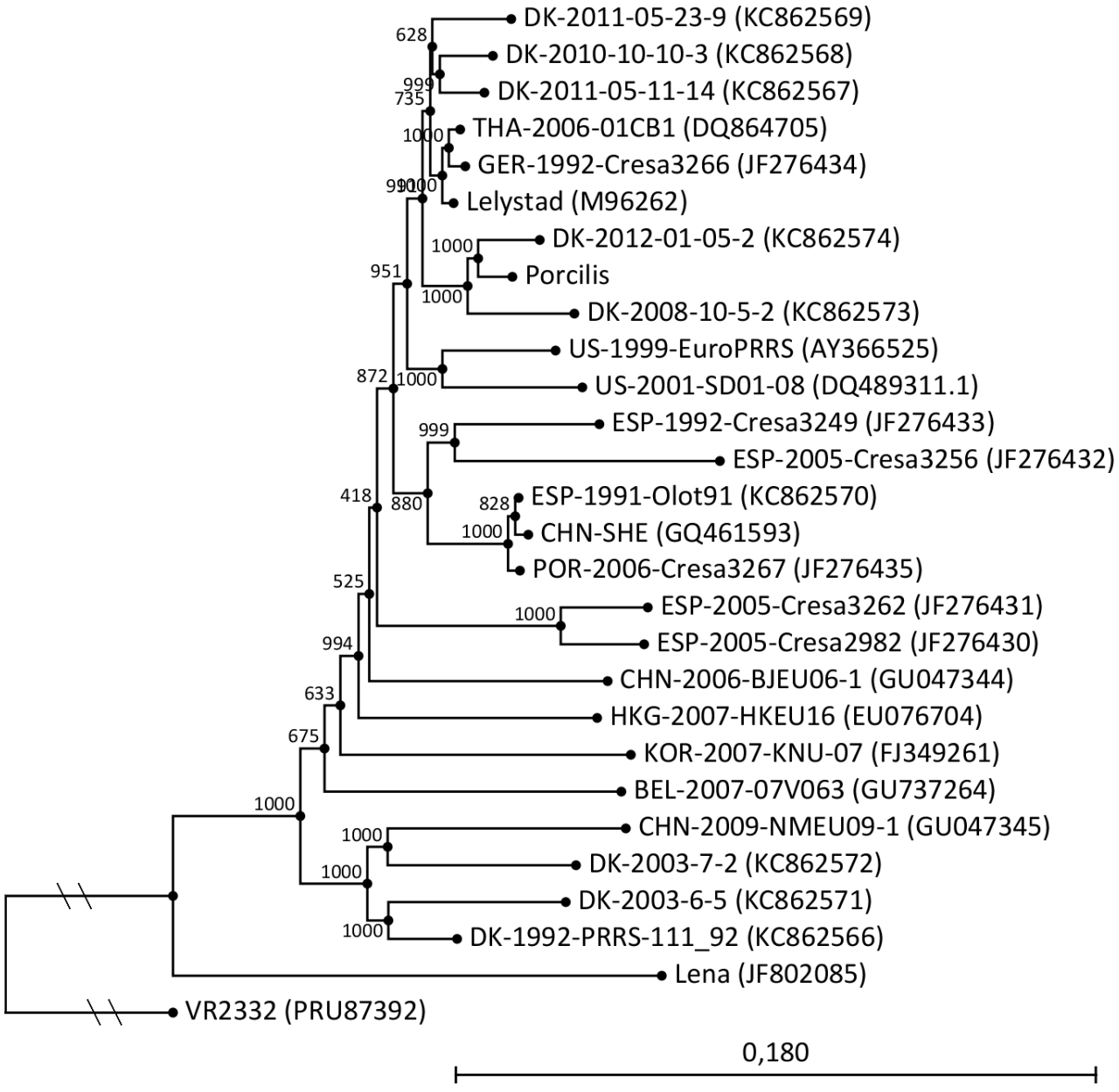
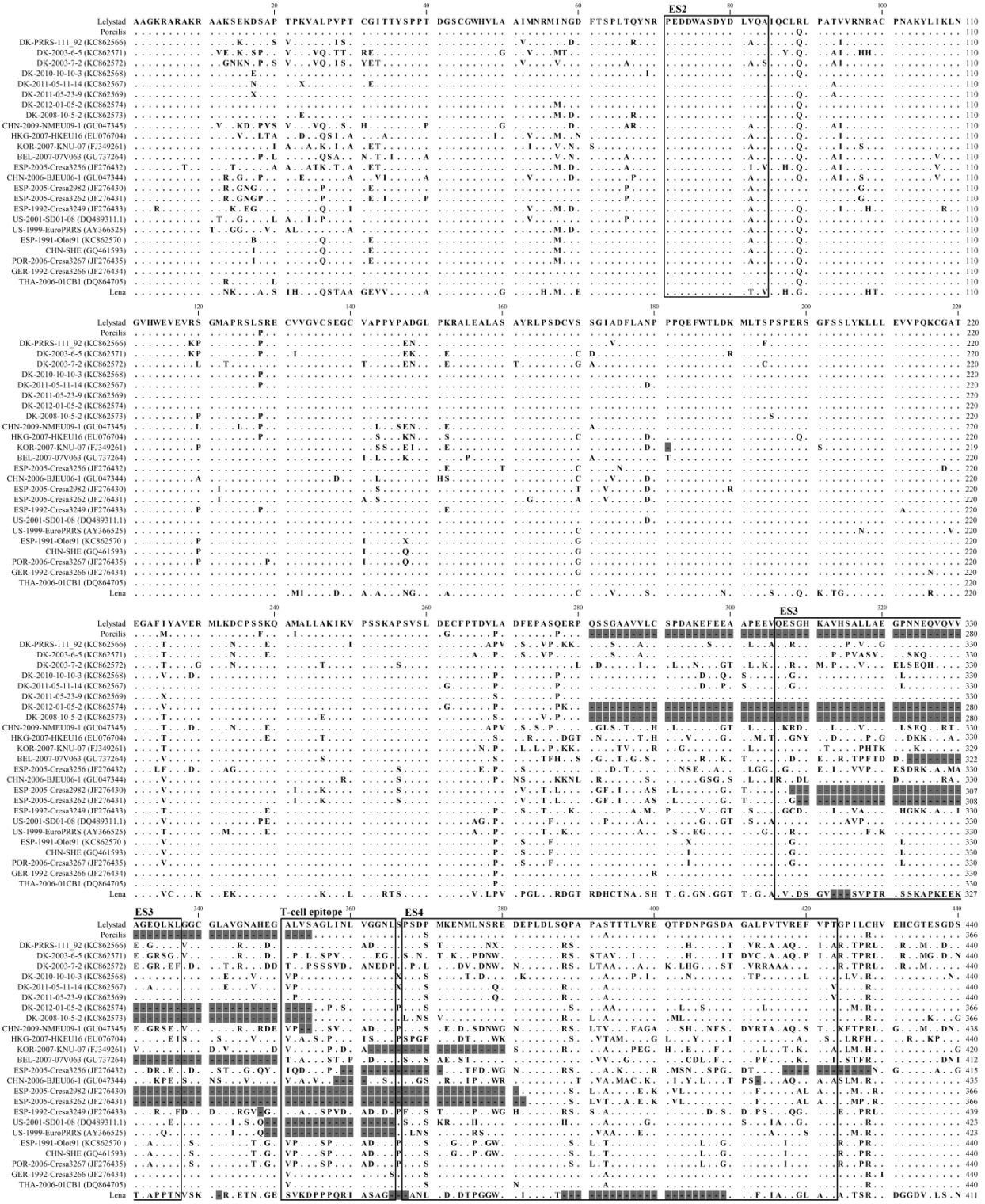


Fig. 6. Alignment of the putative amino acid sequence of nsp2 for PRRSV Type 1. Epitope sites (ES) described by Oleksiewicz (2001) are highlighted in black boxes. Grey squares represents deletions.



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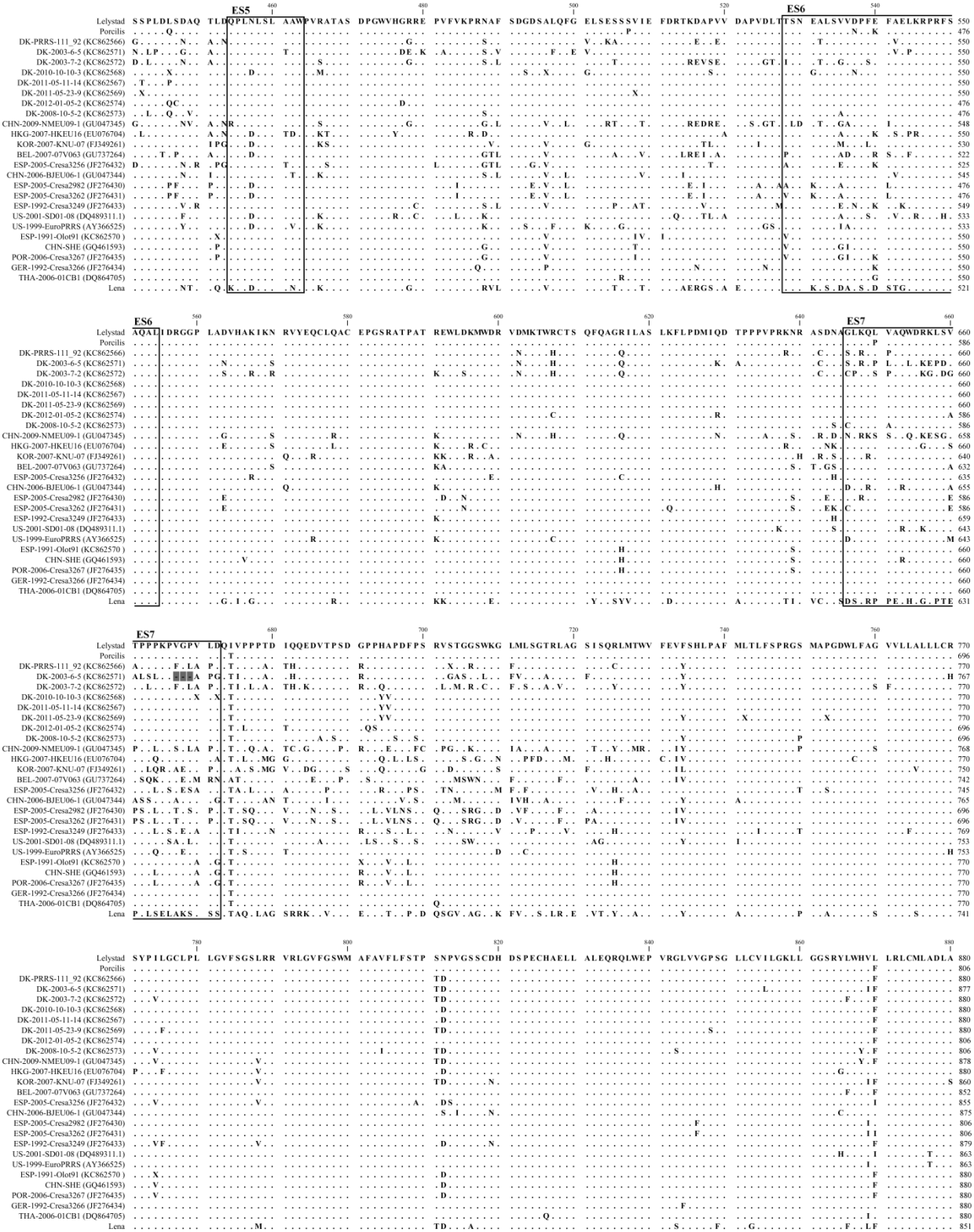

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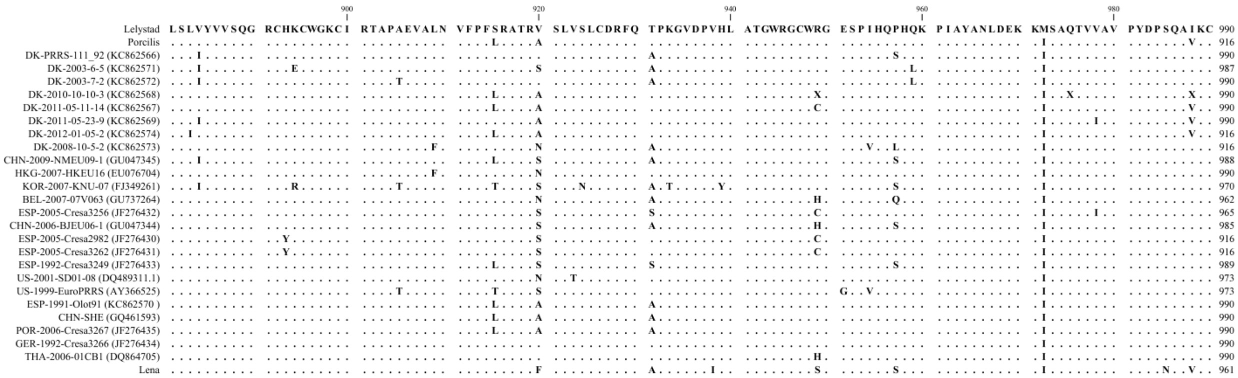
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920
940
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Porcilis
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DK-2003-6-5 (KC862571)
DK-2003-7-2 (KC862572)
DK-2010-10-10-3 (KC862568)
DK-2011-05-11-14 (KC862567)
DK-2011-05-23-9 (KC862569)
DK-2012-01-05-2 (KC862574)
DK-2008-10-5-2 (KC862573)
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ESP-2005-Cresa3262 (JF276431)
ESP-1992-Cresa3249 (JF276433)
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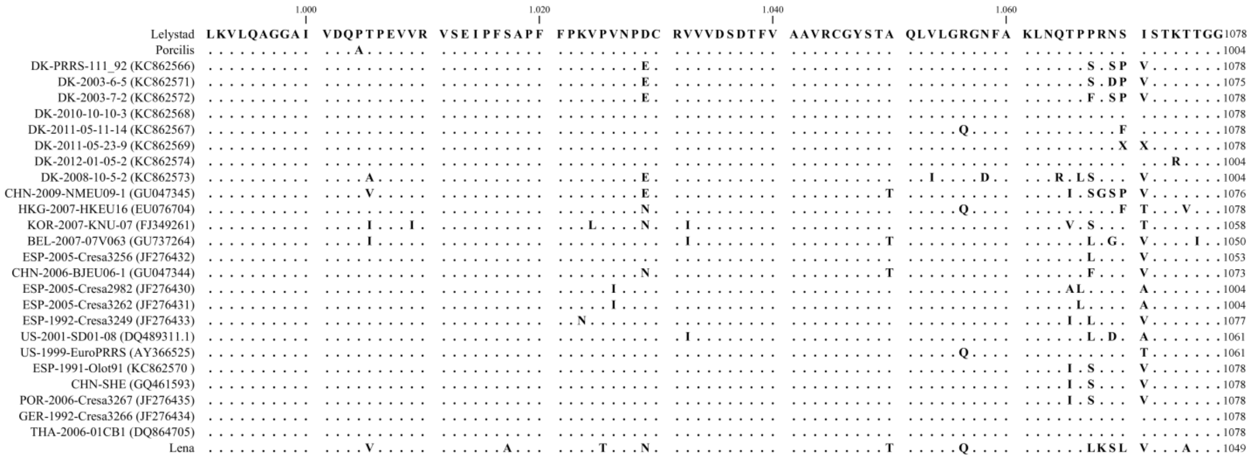
Lena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1.020
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Porcilis
DK-PRRS-111_92 (KC862566)
DK-2003-6-5 (KC862571)
DK-2003-7-2 (KC862572)
DK-2010-10-10-3 (KC862568)
DK-2011-05-11-14 (KC862567)
DK-2011-05-23-9 (KC862569)
DK-2012-01-05-2 (KC862574)
DK-2008-10-5-2 (KC862573)
CHN-2009-NMEU09-1 (GU047345)
HKG-2007-HKEU16 (EU076704)
KOR-2007-KNU-07 (FJ349261)
BEL-2007-07V063 (GU737264)
ESP-2005-Cresa3256 (JF276432)
CHN-2006-BJEU06-1 (GU047344)
ESP-2005-Cresa2982 (JF276430)
ESP-2005-Cresa3262 (JF276431)
ESP-1992-Cresa3249 (JF276433)
US-2001-SD01-08 (DQ489311.1)
US-1999-EuroPRRS (AY366525)
ESP-1991-Olot91 (KC862570)
CHN-SHE (GQ461593)
POR-2006-Cresa3267 (JF276435)
GER-1992-Cresa3266 (JF276434)
THA-2006-01CB1 (DQ864705)
Lena

Fig. 7. Partial amino acid alignment of GP3. (A). N-terminal of GP3. Epitope site ES11 is highlighted with a black box. (B) C-terminal of GP3. Deletions in GP3 are represented with gray rectangles and the putative antigenic site (RKASLSTS) of GP3 is highlighted with a black box.

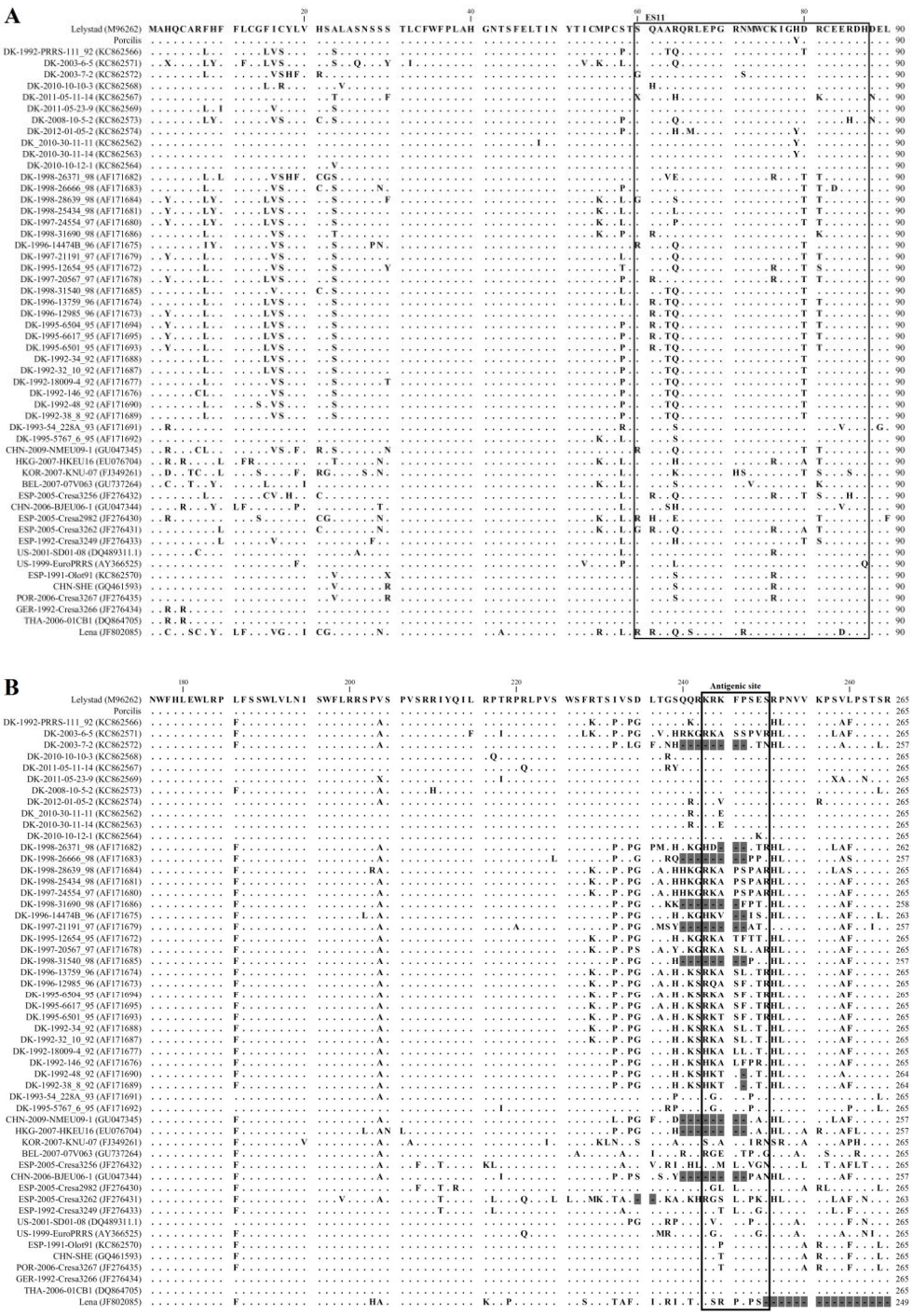
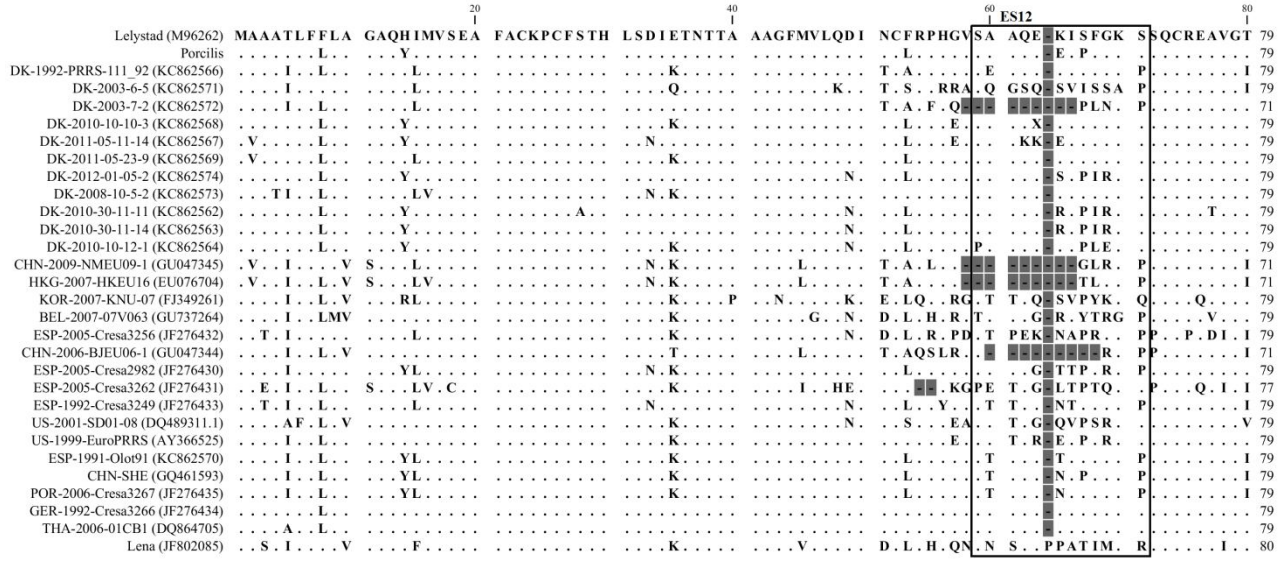


Fig. 8. Amino acid alignment of the N-terminal part of GP4. Deletions in GP4 is represented with gray rectangles and the predicted epitope site 12 (ES12) of GP4 is highlighted with a black box.



Manuscript III

Genetic and antigenic dissection of complete genomes of PRRSV Type 2 viruses isolated in Denmark over a period of 15 years

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(Submitted for publication)

Abstract

Type 2 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) was first detected in Europe in 1996 co-incident with the introduction of a live attenuated vaccine that reverted back to virulence. Since then, only limited ORF5 and ORF7 sequences of Type 2 PRRS viruses have been reported throughout Europe, and these sequences are very similar to the vaccine strain (>98 %). Until now, no complete genomes of Type 2 PRRSV isolated in Europe have been made public. In the present study, the genetic and antigenic diversity of 11 complete genomes and 49 full-length ORF 5 sequences of Type 2 PRRS viruses isolated in Denmark from 2003-2012 were examined. The genetic identity of the 11 complete genomes to the vaccine strain ranged between 93.6 - 99.6 % while the 49 ORF5 sequences examined were 94.0-99.8 % identical to the VR2332 strain. Phylogenetic analysis in a global Type 2 PRRSV framework classified all Danish isolates to a single cluster (sub-lineage 5.1) which comprised strains closely-related to the Type 2 prototype isolate VR2332 and a commercially available modified live vaccine.

Keywords:

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

Genotype 2/Type 2

Full genome/complete genome

Non-structural protein 2 (nsp2)

Amino acid variations

ORF5

1. Introduction

A mystery swine disease causing reproductive failure in sows and severe pneumonia in piglets was first described in North America in the late 1980s and was a few years later also observed in Europe (Keffaber. 1989; Wensvoort et al. 1991). The etiological agent of the disease was found to be viral and finally named Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) based on the clinical signs (Terpstra et al. 1991; Wensvoort et al. 1991; Collins et al. 1992). Isolation and characterization of viruses obtained from the two continents indicated a pronounced degree of genetic heterogeneity with only 50-60 % nucleotide identity (Wensvoort et al. 1991; Collins et al. 1992; Allende et al. 1999). Subsequently, PRRS viruses were divided into two major genotypes named Type 1 for the genotype initially described in Europe and Type 2 for the North American genotype (Allende et al. 1999). Nowadays, both genotypes circulate worldwide and therefore the nomenclature has been changed to Type 1 for the European genotype and Type 2 for the genotype initially found in North America (Fang et al. 2004; Ropp et al. 2004; An et al. 2010; Chen et al. 2011).

PRRSV is an enveloped single-stranded positive-sense RNA virus, belonging to the *Arteriviridae* family within the order *Nidovirales* (Cavanagh. 1997). The genome is 15-15.5 kb long and encodes 10 ORFs (Meulenberg et al. 1993; Wu et al. 2001; Firth et al. 2011; Johnson et al. 2011). ORF5, encoding the glycoprotein GP5, is one of the most variable genes and is therefore often used in phylogenetic analysis (Conzelmann et al. 1993; Key et al. 2001; Shi et al. 2010). A serological survey performed in 1996 documented that Denmark was free of antibodies against Type 2 PRRSV prior to the introduction of a modified live vaccine (Botner et al. 1997; Madsen et al. 1998). Since then, Type 2 PRRSV infections have been sporadically reported throughout Europe, however, ORF5 and ORF7 sequences of European Type 2 PRRSVs are scarce. Apart from a Hungarian Type 2 PRRSV strain very different from all published sequences (Balka et al. 2008), all published European Type 2 viruses shared a high degree of identity to the PRRSV MLV vaccine strain (>98%) which is based on the VR2332 isolate . In 2006, China experienced the emergence of a highly pathogenic strain of PRRSV which had a unique genomic structure (Tian et al. 2007; An et al. 2010). The PRRSV epidemic in China affected more than 2,000,000 pigs with about 400,000 fatal cases. This abrupt occurrence of highly pathogenic PRRSV strains emphasizes the significance of monitoring the diversity of circulating strains around the world both in respect to the sensitivity and specificity of diagnostic tests as well as efficacy of available vaccines.

Today - more than 15 years after the initial introduction - Type 2 PRRSV still causes significant clinical problems in Danish herds. Previously sequenced Danish Type 2 PRRSV isolates were reported to have a high level of identity to the vaccine strain (Madsen et al. 1998; Storgaard et al. 1999). However, since only a few Danish PRRS viruses have been sequenced during the last 15 years, the diversity of circulating Type 2 viruses in Denmark and Europe is unknown. This represents an important void especially since the export of Danish pigs to Eastern and central Europe is significant. For example, in 2012 Denmark exported more than 9 million live pigs whereas the import of breeding animals was much less (n < 100). Despite this limited import of live pigs, it is still possible that foreign PRRSV isolates may be introduced into Denmark by contaminated transport carriers or persons as recently seen in Sweden (Carlsson et al. 2009). Similarly, insight into PRRSV diversity in Denmark is of mutual interest for a range of countries. Accordingly, the main objective of the present study was to close the knowledge gap on the temporal diversity of circulating Type 2 PRRS viruses in Danish pigs. A comprehensive sequence analysis of ORF5 and its gene product, GP5, of Danish Type 2 PRRSV viruses isolated in the years 2003-2012 was performed and compared to the early introduced Danish Type 2 viruses and globally isolated Type 2 viruses. Additionally, for the first time, a thorough genetic and antigenic analysis of 11 complete genome sequences of Type 2 PRRSV isolated in Europe was conducted.

2. Material and methods

2.1. Sample material

Lung tissue, serum, oral fluid, and nasal swabs were obtained from 35 Danish swine herds in the years 2003 to 2012. A number of strains were propagated for one passage in Marc-145 cells using the general cell culture procedure (Kim et al. 1993).

2.2. RNA extraction

Total RNA was extracted from serum, nasal swabs, cell culture supernatant, and lung tissue. Lung tissue was prepared as a 5 % homogenate in RLT buffer (QIAGEN) containing 1 % β-mercaptoethanol (Sigma-Aldrich). RNA was extracted from lung homogenate and nasal swabs using RNeasy Minikit (QIAGEN) according to the manufacturer's instructions. Total RNA from serum and cell culture supernatant was purified using QIAamp Viral RNA Mini Kit (QIAGEN). Elution volume for both extractions methods was 60 µl. The RNA was stored at -80°C until use.

2.3. Real time RT-PCR

Purified RNA was screened for PRRSV using a modification of the Primer Probe Energy Transfer RT-PCR (PriProET-RT-PCR) assay described by Balka et al. (Balka et al. 2009).

2.4. cDNA synthesis and PCR amplification

Full-genome cDNA synthesis was performed by SuperScript® III First-Strand Synthesis System (Invitrogen) following the recommendations by the manufacturer except that the cDNA synthesis step was extended to 90 minutes. A poly(dT) RT-primer was used as cDNA primer (5'-CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT $T(T)_{38}$-3' (Nielsen et al. 2003). PCR amplification of ORF5 and ORF7 was carried out using the AccuPrime™ *Taq* DNA Polymerase High Fidelity Kit (Invitrogen). The PCR mixture was prepared as recommended by the supplier except that the amount of AccuPrime™ Taq High Fidelity was increased to 0.5 µl. The PCR amplification was conducted on a T3 thermo cycler (Biometra) with the following conditions: [94°C for 15 seconds], 45 cycles: [94°C 15 seconds, 55°C for 30 seconds, 68°C for 60 seconds] then finalize with 68°C for 5 minutes and cool down to 4°C. PCR primers for amplifying ORF5 and ORF7 were from Oleksiewicz, 1998 (Oleksiewicz et al. 1998). PCR products were analyzed on 2 % agarose gels (E-gels, Invitrogen).

Long range PCR amplification was performed using the full-genome cDNA as template with AccuPrime™ Taq High Fidelity kit as described by Kvisgaard et al. 2013.

2.5. Cycle sequencing and next generation sequencing

PCR products of the ORF5 and ORF7 amplifications were sequenced by cycling sequencing using the Sanger method (Sanger et al. 1977) with the ORF5 and ORF7 primers used as sequencing primers (Sanger et al. 1977). In total, 39 ORF5 sequences (accession no. KC506625; KC506628-30; KC506632-35; KC506637-41; KC506643-59; KC506661-662; KC506665; KC506667-69; KC506671-72; KC577601) and 45 ORF7 sequences (accession no. KC506674; KC506677-79; KC506681-84; KC506686-90; KC506692-712; KC506714-15; KC506718; KC506720-22; KC506724-26; KC577602-3) were generated.

Full genome sequences were generated by next generation sequencing technologies (NGS) using long range PCR amplicons covering the full genome of PRRSV in two or four fragments as templates. For a detailed description of the procedure and next generation sequencing see Kvisgaard et al. 2013. In short, equimolar concentration of the PCR amplicons covering the full genome of

PRRSV were prepared for sequencing on Roche/454 Genome Sequencer FLX + Titanium (LGC Genomics GmbH, Berlin, Germany and Ion Torrent GPM sequencer (DTU Multi-Assay Core (DMAC), Kgs. Lyngby, Denmark). Additionally, full length sequences of two Danish viruses (DK-2010-10-2-1 and DK-2010-10-7-1) were obtained using the 454 GS Jr. platform. DNA template for these two isolates was generated using a random PCR method previously described (Van Doorsselaere et al. 2011).

2.6. Sequence analysis

Data analysis of ORF5 and ORF7 sequences obtained from the cycle sequencing was carried out using the commercial software CLC Main Workbench v. 6.6.2 (CLC BIO, Arhus, Denmark). Contigs of ORF5 and ORF7 were produced from assembling the raw data obtained from cycle sequencing against the reference sequence VR2332 (PRU87392). Nucleotide and amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log- Expectation) (Edgar. 2004). The ORF5 sequences were classified based on a globally representative ORF5 phylogenetic framework (Shi et al. 2010). The ORF5 phylogenetic tree of the newly sequenced Danish isolates and reference sequences was constructed using a Bayesian Markov chain Monte Carlo (BMCMC) method implemented in MrBayes v3.2 (Ronquist et al. 2012) under settings previously described (Shi et al. 2010).

Mapping of reads obtained from full genome sequencing was performed by the Burrows-Wheeler aligner (BWA) using the Aln algorithm for the Illumina HiSeq2000 data and the bwasw algorithm for Roche 454 FLX and Ion Torrent PGM sequencer data (Kvisgaard et al. 2013). Amino acid sequences were predicted from the nucleotide sequences using CLC Main Workbench v. 6.6.2. Nucleotide and amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log- Expectation). The phylogenetic tree constituting the complete genomes were constructed from Neighbor Joining Algorithm with Bootstrap: 1,000 replicates (CLC Main Workbench v. 6.6.2, CLC BIO, Arhus, Denmark).

Potential N-glycosylation sites were determined for GP2, GP3, GP4, and GP5 using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Using a neural network, N-glycosylation sites were selected when potential above the threshold 0.5 was reached (http://www.cbs.dtu.dk/services/NetNGlyc/output.php).

3. Results

3.1. Analysis of ORF5 and ORF7 sequences

A total of 49 ORF5 and 55 ORF7 nucleotide sequences were obtained from 57 viruses collected in the years 2003-2012. The lengths of the ORF5 and ORF7 sequences were 603 and 372 nucleotides, respectively. The viruses DK-2011-30-3-13, DK-2011-30-3-15, and DK-2011-30-3-20 collected simultaneously from the same pig herd, were almost identical and had a 3 nucleotide deletion in ORF5 (corresponding to residues114-116 in Ingelvac PRRS MLV). This deletion was also present in two non-European viruses, one from Thailand and one from the USA (THA-2002-02SP3 and US-ISU3927) however, these isolates shared only 86.8% and 92.3 % identity in ORF5 to these three Danish sequences. Among the Danish sequences, the pairwise nucleotide identity was 90.9-100% and 93.0-100.0 % for ORF5 and ORF7, respectively. The pairwise nucleotide identity of the Danish sequences to the Ingelvac PRRS MLV vaccine strain was in the range 94.0-99.8 % and 94.6-100 % in ORF5 and ORF7, respectively. Phylogenetic analysis of ORF5 in a globally representative context (Shi et al. 2010) showed all the Danish viruses to be closely related to the cluster of VR2332-like or vaccine-like strains i.e. sub-lineage 5.1 (Fig. 1). Similar comparisons solely between Danish isolates indicated a maximum nucleotide non-identity of 9.1 %.(Fig. 1). Pairwise nucleotide comparisons based on a partial ORF5 region (nt91-522) between Danish Type 2 PRRS viruses and two Hungarian Type 2 PRRS viruses from 2005 and 2006 (Balka et al. 2008) showed only 84.2-88.2 % identity (data not shown).

3.2. Antigenic analysis of the deduced amino acid sequence of the major glycoprotein 5, GP5

Examination of the deduced amino acid sequences of ORF5, GP5, showed that 144 positions out of 200 were conserved in all 49 Danish sequences (72 % conserved sites). Twenty-four positions varied in more than one virus, and 7 positions varied in more than 10 viruses. The most variable position was at aa34 where 34 viruses varied. Six different amino acid residues were seen at this site. Interestingly, position aa34 is a putative N-glycosylation site in Type 2 PRRS viruses. The cysteine residue at position aa48 thought to be involved in heterodimer formation with the M protein (Mardassi et al. 1996) was conserved in all the Danish sequences. The decoy epitope (A/VLA/VN motif) at position aa27-30 (Ostrowski et al. 2002) is located in a hypervariable region of GP5 and this motif was conserved in the majority of the Danish viruses (Fig. 2). The neutralizing

epitope of GP5 located in the region aa37-45 (SHL/FQLIYNL) is known to be highly conserved, which also was seen for the Danish viruses (Fig. 2) (Ostrowski et al. 2002).

Potential N-glycosylation sites were observed at seven different positions: N30, N32, N33, N34, N35, N44, and N51 (Table 1). Using a neural network, N-glycosylation sites were predicted to be utilized if the threshold was above 0.5. The result of these network predictions, revealed 8-9 different putative glycosylation patterns among the Danish viruses (Table 1). N-glycosylation sites variations were located mainly between aa30-35 near the highly variable region, including the highly variable site at position aa34 as mention previously. The N44 and N51 N-glycosylation sites were conserved in all but one sequence, where N44->D44 but the N51 glycosylation site was conserved in this virus (DK-2012-10-1-5). The most common N-glycosylation pattern was N30, N33, N44, and N51 which 34 viruses harbored, however, in 14 of these sequences N30 did not reach the potential threshold of 0.5 and therefore was not predicted to be N-glycosylated by the neural network (glycosylation pattern 2, Table 1).

3.3. Genetic analysis of 11 complete Danish PRRSV Type 2 genomes

Eleven complete genomes of Type 2 PRRSV (Kvisgaard et al. 2013) isolated from Danish pig herds in the years 1997 to 2012 were aligned and analyzed. The genome lengths varied from 15,396-15,408 nucleotides (excluding the Poly(A) tail) and the pairwise nucleotide identity among the Danish viruses was 90.6-99.3 % and they were 93.6-99.6 % identical to the vaccine strain Ingelvac PRRS MLV (Table 2). DK-2008-10-1-3 and DK-2010-10-1-2 were clearly different from the majority of Danish isolates in that the pairwise nucleotide identities to the other Danish sequences were 92.8-95.3 % and 91.1-93.6 %, respectively. These two isolates were only 90.6 % identical. Furthermore, DK-2010-10-1-2 (sub-lineage 5.2) shared only 93.6 % nucleotide identity to Ingelvac PRRS MLV vaccine (sub-lineage 5.1) making it the most diverse of the Danish viruses at the genetic level which was supported by phylogenetic classification as well. A phylogenetic tree was constructed with the complete Danish sequences and Type 2 sequences isolated in North America and China (Fig. 3). Despite the fact that the Danish isolates varied up to 10% of genomic sites, they all grouped together in a cluster which also included the vaccine strain, VR-2332 and three other US strains isolated in the mid-1990s. This grouping largely mirrored the phylogenetic clustering (lineage 5) described earlier using ORF5 only.

Table 3 lists the pairwise nucleotide and deduced amino acid identity of the non-structural proteins (nsp) and structural proteins of the Danish viruses. The average similarity of the different

ORFs ranged from 94.9 ± 2.9 % for ORF1a to 97.8 ±1.5 % for ORF6. The low average similarity of ORF1a was mainly due to the low similarity in the coding regions of NSP1β and NSP2 among the Danish sequences which were in the range of 85.1-99.3 % and 86.6-98.9 %, respectively. The similarity of the protein products, nsp1β and nsp2, was also low (80.8-99.5 % and 94.0-98.2 %), thus indicating that most mutations in this region are non-synonymous.

3.4. Examination of the non-structural protein 2 (nsp2) of the Danish PRRSV Type 2 viruses

Fig. 4 shows an alignment of the deduced nsp2 amino acid sequences of 11 Danish viruses together with three North American isolated Type 2 PRRS viruses reported to be highly virulent (Han et al. 2006; Gauger et al. 2012) and three Chinese PRRSV Type 2 viruses, one high pathogenic (GDBY1), one low pathogenic (GDQJ), and one (BJ-4) very similar to Ingelvac PRRS MLV (99.7 % nucleotide identity) (Yan et al. 2007; Li et al. 2010). The virus, BJ-4, has also been used for B-cell epitope identifications in nsp2 (Yan et al. 2007). Alignment of the predicted amino acid sequence of nsp2 of the Danish viruses with the vaccine strain revealed that several of the Danish viruses harbored deletions resulting in variation of the length of nsp2 from 1174 to 1096 amino acids residues. One virus harbored a 4 amino acid deletion following the amino acid at position 793 and five other viruses harbored a 3 amino acid deletion at the position corresponding to aa593-595 in nsp2 of Ingelvac PRRS MLV. One of the viruses harboring the 3 amino acid deletion was the virus DK-2010-10-1-2 and this virus also had a 19 amino acid deletion corresponding to the position aa498-516 in Ingelvac PRRS MLV nsp2. Thus, this virus encoded the shortest nsp2 of the Danish viruses with an amino acid length of 1174 residues. All the deletions observed in the Danish viruses were located at the hyper variable region between aa150-850 of nsp2. The nsp2 protein is known to be the most diverse protein of all the proteins encoded by PRRSV, nevertheless, the cysteine protease domain (PL2) located in the region aa46-146 of nsp2 is known to be conserved within the same genotype. Indeed, this region was also highly conserved in the Danish viruses with only 17 substitution sites out of 101positions while the North American and Chinese viruses had only 16 and 9 substitution sites, respectively, in comparison to the Ingelvac PRRS MLV nsp2 sequence. The amino acid residues forming the putative catalytic triad C54, D88, and H123 (Han et al. 2009), were conserved in all the Danish viruses as well as in the American and Chinese viruses. All 6 putative B-cell epitopes identified by Yan et al. (2007) are highlighted in Fig. 4. Epitope sites SP1, SP2, and SP4 were highly conserved among the Danish viruses, whereas epitope sites SP5, SP6, and SP8 showed a high proportion of variable sites (60 %, 64 %, and 69 %, respectively).

Comparison of the Danish nsp2 sequences to the vaccine strain showed high similarity for 7 of the viruses (DK-1997-19407B, DK-2004-1-7-Pl, DK-2010-10-2-1, DK-2010-10-4-1, DK-2010-10-7-1, DK-2011-030311-1, and DK-2012-01-11-3) with 96.2-99.3 % amino acid identity and lower similarity for 4 viruses (DK-2003-2-3, DK-2004-2-1, DK-2008-2-1, and DK-2010-10-1-2) with amino acid identity in the range of 89.0-92.5 %.

3.5. Antigenic analysis of the minor glycoproteins, GP2, GP3, and GP4

The pairwise amino acid identities of the minor glycoproteins, GP2, GP3, and GP4 among the Danish sequences are listed in Table 3. GP3 was shown to be the most diverse structural protein among the Danish Type 2 viruses with amino acid identity of 90.9-99.2 %. Linear B-cell epitopes have been identified in all 3 minor glycoproteins using Pepscan technologies using the sequence of the virus strain NVSL 97-7895 (de Lima et al. 2006). The GP2 comprised two putative linear B-cell epitopes at amino acid positions aa41-55 and 121-135, respectively (Fig. 5). These regions were highly conserved among the Danish viruses; however, the epitope located at the region aa41-55 harbored 4 substitutions sites compared to the NVSL 97-7895 strain. For GP3, 4 overlapping consecutive linear B-cell epitopes have been identified (aa61-75, aa71-85, aa81-95, and aa91-105) (de Lima et al. 2006). Even though GP3 was the most diverse structural protein among the Danish Type 2 viruses, the 4 epitope sites were all highly conserved with only 8 substitution sites distributed throughout all 4 epitope regions (aa61-105) when compared to the NVSL 97-7895 strain GP3 sequence (Fig. 6). The putative epitope site in GP4 at position aa51-65 was not conserved among the Danish viruses with 10 substitution sites out of a possible of 15 (Fig. 7).

Examination of potential N-glycosylation sites revealed 2 putative N-glycosylation sites at N178 and N184 in GP2 (Fig. 5), 7 putative N-glycosylation sites (N29, N42, N50, N131, N152, N160, and N195) in GP3 (Fig. 6), and 4 putative N-glycosylation sites (N37, N84, N120, and N130) in GP4 (Fig. 7). The result of nine neural networks predicted all putative N-glycosylation sites in GP2 and GP4 to be N-glycosylated however N37 in GP4 did not score a unanimous vote from the jury network to be N-glycosylated. For GP3 all 7 putative N-glycosylation sites were predicted to be N-glycosylated in all the Danish viruses except for virus DK-2008-10-1-3 where the potential at this position did not reach the threshold of 0.5. A unanimous vote from the jury predicted N-glycosylation at N195 however this position is believed to be located in the transmembrane region of GP3, hence unlikely to be glycosylated (Dokland. 2010).

The cysteine residues important for protein folding and function was 100 % conserved in all the minor structural proteins of the examined Danish viruses.

4. Discussion

We report here for the first time the sequences of complete genomes obtained from Type 2 PRRS viruses isolated in Europe. With the 11 complete genomes sequenced from viruses isolated in the years 1997-2012 (Kvisgaard et al. 2013), the evolution of Type 2 PRRSV since its introduction 15 years ago in Denmark is now well described. The virus DK-1997-19407B was isolated about 6 months after the Danish PRRSV Type 2 vaccination program first started in the fall of 1996. The virus was obtained from a herd that had not been vaccinating against PRRSV Thus, since it is regarded as very unlikely that another VR2332-like virus was introduced into Denmark in the fall of 1996 coincidentally with the start of the vaccination program, this virus must incorporate the genetic changes incurred by the PRRS vaccine virus while spreading between herds and can be regarded as the historical European Type 2 PRRSV reference strain (Nielsen et al. 2001). In the present study, we sequenced the complete genome of this reference strain and showed that it was 99.4 % identical to the vaccine strain and to two other Type 2 viruses isolated in the US in 1995 and 1997, respectively. These findings support epidemiological data indicating that Type 2 PRRSV was likely introduced into Denmark by the MLV vaccine. This view is further sustained by the negative outcome of a serological testing of 2159 sows performed prior to the introduction of the vaccine in the autumn of 1996 using a type specific immunoperoxidase monolayer assay (IPMA) (Botner et al. 1994; Sorensen et al. 1997).

The sudden emergence of more pathogenic PRRS viruses in North America (Han et al. 2006; Gauger et al. 2012), Asia (Tian et al. 2007; An et al. 2010) and Eastern Europe (Karniychuk et al. 2010; Weesendorp et al. 2013; Morgan et al. 2013) emphasize the importance of monitoring the genetic diversity of PRRSV. Despite this obvious need, there has been only a limited focus on the genetic diversity of Type 2 PRRSV in Europe, perhaps because the few viruses that were analyzed were found to be very similar (> 98 %) to the Ingelvac PRRS MLV vaccine in ORF5 and ORF7 (Stadejek et al. 2013). This has led to the perception that all Type 2 viruses in Europe are closely related to the attenuated vaccine strain and are only minor contributors to clinical PRRS in Europe. In Hungary, however, where the Ingelvac PRRS MLV vaccine has not been used (Balka et al. 2008), two Type 2 PRRS viruses were isolated in 2005 and 2006. First it was believed that these viruses was introduced through infected breeding animals imported from Denmark, however, later it

was shown that they shared 91 % identity to a Canadian strain indicating that they may have been introduced by other sources (Balka et al. 2008). Pairwise nucleotide comparison of the partial ORF5 sequences of the Hungarian viruses to the 49 Danish viruses examined in our study revealed that they only shared 84.2-88.2 % nucleotide identity and therefore support that these isolates did not originated from Denmark.

The majority of the 11 complete genomes showed high level of similarity to the vaccine strain with pairwise nucleotide identity of 98.1-99.6 %. This similarity correlated well with the diversity in ORF5 of the Danish viruses where all the ORF5 sequences clustered along with the vaccine strain in lineage 5 (Shi et al. 2010). Interestingly, four viruses isolated in 2003, 2004, 2008, and 2010 showed a higher level of diversity to the vaccine strain with 93.6-96.1 % complete genome similarity, however, they still clustered within the lineage 5 in the phylogenetic analysis. Based on the ORF5 nucleotide sequences obtained from all Danish viruses isolated between 2003-2012, the diversity of the Danish viruses to the vaccine strain was in the range of 94.0-99.8 %, which is a larger range than the diversity of most European Type 2 viruses (> 98 % similarity to the vaccine strain) (Greiser-Wilke et al. 2010; Stadejek et al. 2013).

Notwithstanding insights based on ORF5, the examination of limited genomic regions may not be sufficient for a more complete understanding of PRRSV sequence heterogeneity, as illustrated by the non-structural protein coding regions NSP1β and NSP2 of the Danish viruses that showed a very broad identity range to the vaccine strain in the range of 85.1-99.3 % and 86.6-98.9 %, respectively.

At the genomic level, the isolates DK-2008-10-1-3 and DK-2010-10-1-2 exhibited the greatest divergence with pairwise nucleotide identity to the other Danish genome sequences in the range of 92.8-95.3 % and 91.1-93.6 %, respectively and shared only 90.6 % nucleotide similarity between each other. DK-2010-10-1-2 was the virus with the shortest genome constituting 15,342 nucleotides with a discontinuous deletion in the coding region of NSP2 of 57 plus 9 nucleotides (corresponding to 19 plus 3 amino acids in the deduced amino acid sequence of nsp2, Fig. 4). The consecutive 9 nucleotide deletion was also seen in 4 other viruses examined in this study. Although some of these viruses were sequenced from RNA extracted from cell culture supernatant, the deletion is naturally occurring since it was also found in partial NSP2 sequences obtained from the corresponding primary material (data not shown). During screening of partial NSP2 sequences, several viruses (from 2003, 2010 and 2011) were found to harbor the same deletion (data not shown). Deletions are often seen in the NSP2 coding part of the PRRSV genome (Ropp et al. 2004; Han et al. 2006), and

also in high virulent strains such as the MN184A and MN184B viruses and all the high pathogenic viruses isolated in China during the outbreak in 2006-2009 (An et al. 2010). Based on these findings, one might presume that deletions in NSP2 were linked to high virulence of the virus. However, a low pathogenic virus, GDQJ (GQ374441), isolated in 2008 was shown to harbor the exact same deletion as the high pathogenic Chinese viruses (Li et al. 2010). Additionally, using chimeric viruses, the NSP2 deletion in the 2006 Chinese outbreak of PRRS was shown not to be linked to virulence (Zhou et al. 2009). In this sense, the deletions found in NSP2 of the Danish viruses can be regarded as an 'epidemiological marker' and not as a marker of virulence.

The nsp2 is the viral protein containing the highest frequency of B-cell epitopes (Oleksiewicz et al. 2001; de Lima et al. 2006; Yan et al. 2007) and the humoral antibody response to nsp2 is more pronounced than towards any other PRRSV protein. However, most of the antibodies are non-neutralizing (Han et al. 2007; Johnson et al. 2007; Brown et al. 2009). Analysis of the Danish viruses showed that epitope sites SP1, SP2, and SP4 identified by Yan et al. (2007) were highly conserved among the Danish viruses, whereas epitope sites SP5, SP6, and SP8 showed a high proportion of variable sites of 60 %, 64 %, and 69 %, respectively (Fig. 4). De Lima et al. (2006), identified 18 B-cell epitopes in nsp2 of a North American virus (NVSL 97-7895, acc. no. AY545985), but none of these epitopes were conserved in the Danish nsp2 sequences probably as a consequence of the high level of amino acid variation of nsp2 (amino acid comparison of the North American nsp2 sequence to the Danish nsp2 sequences were 77.5-82.7 % identity). The North American virus, NVSL 97-7895 was also used for screening of linear B-cell epitopes in the structural proteins and the epitopes identified in GP2 and GP3 was highly conserved among the Danish viruses and to the North American strain although the overall amino acid variation was high in all three minor glycoproteins compared to the Danish viruses. The B-cell epitope identified in GP4 at position aa51-65 were highly variable with 10 substitutions out of 15 possible. This fit well with previous findings that this protein only reacted with a small fraction of tested post-infection sera (de Lima et al. 2006). Taken together these results indicated that some - but not all - epitopes present in the nonstructural and structural proteins of Type 2 PRRSV are prone for changes – which again indicate that antibodies generated against some epitopes may have no impact on virus survival. In general, comparisons of full-length genomes provide valuable information on conserved and variable regions and thereby can reveal new targets for immune intervention, the design of new vaccines and for the development of diagnostic tests.

The N-glycosylation of GP5 may be critical for proper functioning of the protein as N-glycosylation, in general, is important for correct folding, targeting, and biological activity of proteins (Ansari et al. 2006). In this study, potential N-glycosylation sites (N-X-S/T-X, X ≠ P) were observed at seven different positions in GP5: N30, N32, N33, N34, N35, N44, and N51. The result of a neural networks predictions, lead to 8-9 different putative glycosylation patterns among the Danish viruses, with the N-glycosylation of N30, N33, N44, and N55 the most abundant pattern. However, even though the N-glycosylation motif at N30 was present in 14 sequences the potential for glycosylation did not reach the threshold of 0.5, hence not predicted to be N-glycosylated. The position N30 is located in the C-terminal part of the putative signal peptide and glycosylation at this position may not have any influence on the mature protein, but may contribute to other biological functions such as immune evasion. The N-glycosylation at position N44 has previously been shown to be important for infectious virus production (Ansari et al. 2006) and only one of the glycosylation patterns found among the Danish GP5 sequences failed to predict this site to be glycosylated because the N44 was substituted to a D44 and the N-glycosylation motif was lost.

Of the 11 complete genomes examined in this study, all of the putative N-glycosylation motifs in the minor glycoproteins GP2, GP3, and GP4 were present and predicted to be N-glycosylated which strongly indicates that the glycosylation of these motifs are important for the functioning of respective proteins.

In conclusion, this study presents for the first time the examination of complete genomes obtained from Type 2 PRRS viruses isolated in Europe. From the complete genomes it was revealed that there was an overall high diversity between the Danish viruses (90.6-99.3 %) and one virus harbored only 93.6 % similarity to the vaccine strain. These results indicate that there has been a pronounced genetic divergence among the Danish Type 2 PRRSVs (sub-lineages 5.1 and 5.2) but the data do not support multiple introductions of different Type 2 strains into Denmark which fits well with the limited import of living animals and semen into Denmark. Lastly, Type 2 viruses in Denmark seem to induce more severe clinical signs compared to other European countries but share relatively close evolutionary relationship in ORF 5 to Type 2 viruses found in other European countries. . This could indicate that the genetic determinants of viral virulence lie outside ORF5 and emphasize that the generation of more complete genome sequences of European Type 2 isolates should be prioritized.

5. Acknowledgement

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 245141 (New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia (PoRRSCon) coordinated by Prof. H. Nauwynck) and the COST Action FA902: Understanding and combating porcine reproductive and respiratory syndrome in Europe (EuroPRRS.net).

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Table 1.

Glycosylation pattern in GP5 of the Danish PRRSV Type 2 viruses isolated in the years 2003-2012 (n = 49)

N-glycosylation pattern	N-glycosylation sites in GP5							No. of sequences	% of total
	N30	N32	N33	N34	N35	N44*	N51		
1	+		+			+	+	20	41
1	+**		+			+	+	14	29
2	+				+	+	+	6	12
3			+			+	+	2	4
4		+				+	+	2	4
5				+	+	+	+	2	4
6						+	+	1	2
7	+		+	+		+	+	1	2
8	+		+	+			+	1	2

*When this N-glycosylation site was present, all viruses scored a 9/9 jury agreement from the neural network.
**The N-glycosylation site is present in the sequence, but the potential for glycosylation did not reach the threshold of 0.5.

Table 2.

Overview of the 11 Danish Type 2 PRRSV complete genomes

Virus	Accession no.	Collection year	Clinical signs (herd)	Sequencing material	Genome length*	Nt identity%**
DK-1997-19407B	KC862576	1997	Stillborn piglet***	Marc-145 isolate	15,396	99.4
DK-2003-2-3	KC862584	2003	-	Marc-145 isolate	15,408	96.1
DK-2004-2-1	KC862585	2004	-	Marc-145 isolate	15,408	96.0
DK-2004-1-7-Pl	KC862578	2004	-	Marc-145 isolate	15,408	99.4
DK-2008-10-1-3	KC862582	2008	Reproductive failure	Lung homogenate	15,408	95.4
DK-2010-10-1-2	KC862579	2010	Respiratory	Lung homogenate	15,342	93.6
DK-2010-10-7-1	KC862580	2010	Respiratory	Marc-145 isolate	15,399	98.8
DK-2010-10-2-1	KC862581	2010	Respiratory	Marc-145 isolate	15,399	99.6
DK-2010-10-4-1	KC862583	2010	Respiratory	Lung homogenate	15,399	98.1
DK-2011-030311-1	KC862577	2011	High fever	Lung homogenate	15,408	99.3
DK-2012-01-11-3	KC862575	2012	Respiratory/ reproductive failure	Lung homogenate	15,399	98.3

*Excluding the Poly(A) tail, **Pairwise nucleotide identity to Ingelvac PRRS MLV (EF484033), *** This virus was obtained from a non-vaccinated herd about 6 months after the Danish vaccination program had started in the fall 1996.

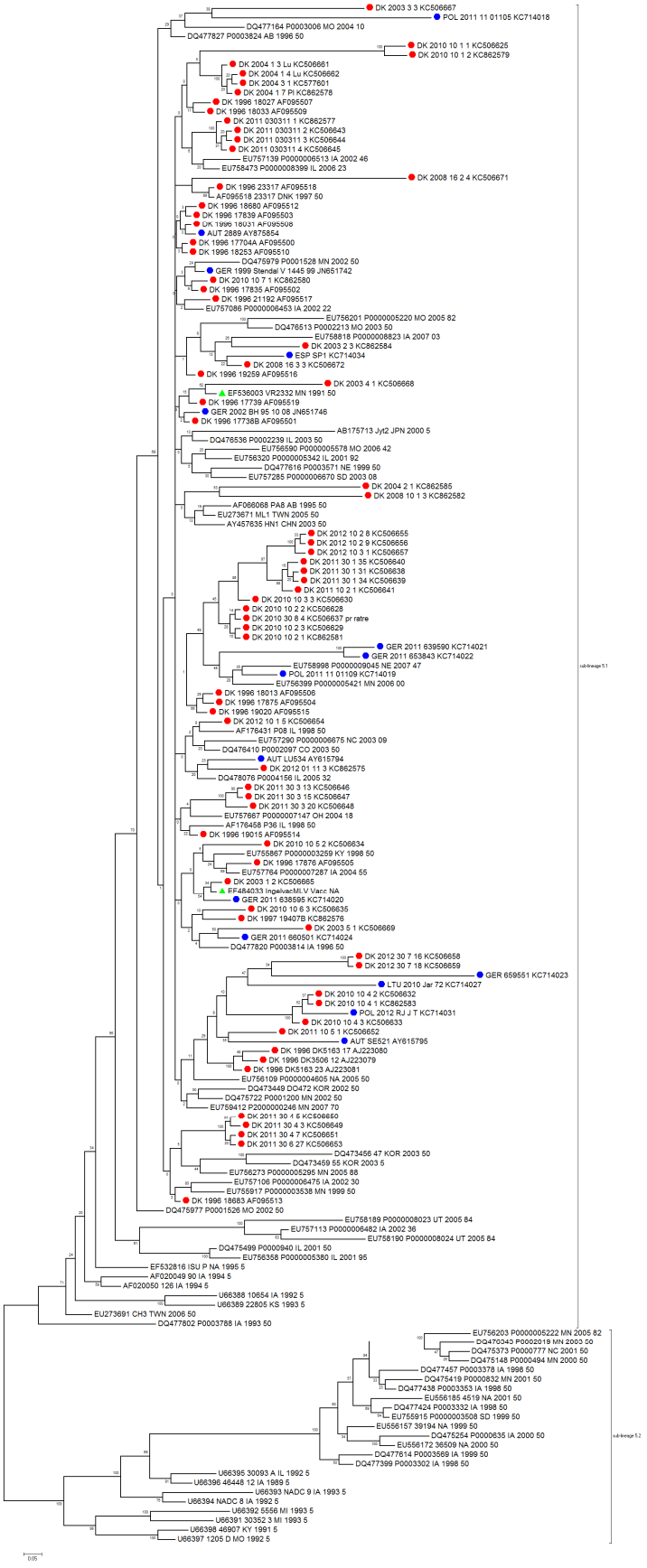
Table 3.

Pairwise nucleotide and amino acid identity range of the 11 Danish PRRSV Type 2 field strains and identity range to Ingelvac PRRS MLV (EF484033). The predicted pp1a and pp1ab protein cleavage sites are from Fang and Snijder, 2010.

ORFs	Length (nt)	% identity range (nt)	Avg. nt identity % (mean ± sd)	% identity range to Ingelvac (nt)	Proteins	Length (aa)	% identity range (aa)	Avg. aa identity % (mean ± sd)	% identity range to Ingelvac (aa)
ORF1a	7446, 7500, 7503, 7512	88.8-99.2	94.9 ± 2.9	92.2-99.6	**pp1a**	2481, 2499, 2500, 2203	89.2-99.2	94.6 ± 2.8	92.4-99.6
NSP1α	540	92.8-99.6	96.9 ± 1.8	95.0-99.8	**nsp1α**	180	96.1-100.0	97.9 ± 1.0	97.2-100.0
NSP1β	609	85.1-99.3	93.8 ±3.6	91.0-99.5	**nsp1β**	203	80.8-99.5	90.8 ± 4.7	87.2-99.5
NSP2	3522, 3576, 3579, 3588	86.6-98.9	94.1 ± 3.4	90.6-99.4	**nsp2**	1174,1192, 1193, 1196	84.0-98.2	92.4 ± 4.0	89.0-99.3
NSP3	690	90.7-99.6	95.8 ± 2.4	94.5-99.9	**nsp3**	230	94.8-100.0	97.9 ± 1.3	97.0-100.0
NSP4	612	90.9-100.0	95.9 ± 2.6	93.8-100.0	**nsp4**	204	94.1-100.0	97.6 ± 1.7	95.6-100.0
NSP5	510	88.6-99.0	95.4 ± 2.6	92.6-99.4	**nsp5**	170	94.1-100.0	97.1 ± 1.6	95.9-100.0
NSP6	48	83.3-100.0	95.7 ± 4.6	87.5-100.0	**nsp6**	16	93.8-100.0	98.9 ± 2.4	93.8-100.0
NSP7α/β	777	90.9-99.5	96.2 ± 2.3	94.2-99.9	**nsp7α/β**	259	95.4-100.0	98.0 ± 1.4	96.9-100.0
NSP8	138	93.5-100.0	97.2 ± 1.9	95.7-100.0	**nsp8**	45	95.6-100.0	98.9 ± 1.3	97.8-100.0
ORF1b	4506	92.5-99.6	96.7 ± 1.9	94.8-99.7	**pp1b**	1501	97.4-99.8	98.7 ± 0.6	98.1-99.9
NSP9	2052	92.6-99.7	96.8 ± 1.8	95.3-99.8	**nsp9**	684	97.4-99.9	98.7 ± 0.7	98.0-100.0
NSP10	1323	92.5-99.6	96.6 ± 2.0	94.9-99.6	**nsp10**	441	96.6-100.0	98.8 ± 0.9	97.3-99.8
NSP11	669	90.6-99.9	96.4 ± 2.4	93.1-99.7	**nsp11**	223	97.3-100.0	99.1 ± 0.8	98.2-100.0
NSP12	462	92.2-99.8	96.1 ± 1.9	94.6-99.8	**nsp12**	153	94.8-99.4	97.4 ± 1.2	96.7-100.0
ORF2	771	91.1-99.7	96.3 ± 2.2	93.8-99.6	**GP2**	256	91.0-99.6	96.3 ± 2.3	92.2-99.2
ORF2b	222	93.7-100.0	97.4 ± 1.9	95.1-99.6	**E**	73	91.8-100.0	96.9 ± 2.0	93.2-98.6
ORF3	765	92.3-99.5	96.4 ± 1.9	94.0-99.6	**GP3**	254	90.9-99.2	95.9 ± 2.2	92.1-98.8
ORF4	537	93.7-99.6	97.1 ± 1.6	95.0-99.1	**GP4**	178	92.1-100.0	96.4 ± 2.3	94.4-99.4
ORF5	603	91.0-99.2	96.0 ± 2.0	94.4-99.3	**GP5**	200	91.5-98.5	95.2 ± 1.8	93.5-99.0
ORF5a	156	91.7-98.7	95.7 ± 1.6	95.5-99.4	**ORF5a protein**	51	88.2-100.0	95.9 ± 2.5	94.1-100.0
ORF6	525	94.3-100.0	97.8 ± 1.5	96.0-99.6	**M**	174	97.7-100.0	98.9 ± 0.6	98.3-99.4
ORF7	372	93.0-100.0	97.7 ± 1.9	94.6-100.0	**N**	123	95.9-100.0	98.9 ± 1.3	97.6-100.0

Next page:

Fig. 1. Phylogenetic analysis of the Danish ORF5 sequences in a globally representative context. All the Danish sequences are marked with a red dot, all European sequences are marked with a blue dot, and the vaccine strain and the Type 2 protogenotype VR2332 are marked with green triangles. All the Danish ORF5 sequences were closely related to the cluster of VR2332-like or vaccine-like strains i.e. sub-lineage 5.1. The tree was constructed using a BMCMC method implemented in MrBayes v3.2.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DK 2008 16 3 3 KC506672
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DK 2003 4 1 KC506668
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GER 2002 BH 95 10 08 JN851746
DK 1996 17738B AF095501
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DK 2012 10 3 1 KC506657
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U66391 30352 3 MI 1993 5
U66398 46907 KY 1991 5
U66397 1205 D MO 1992 5
0.05

Fig. 2. A histogram showing the number of substitutions found at each amino acid position in GP5 of 49 Danish isolates. 'Empty' positions represent conserved amino acids. The seven potential N-glycosylation sites at aa30, aa32, aa33, aa34, aa35, aa44, and aa51 are marked with *. Decoy epitope at position aa27-30 and the Neutralizing epitope at position aa37-45 are marked with black bars.

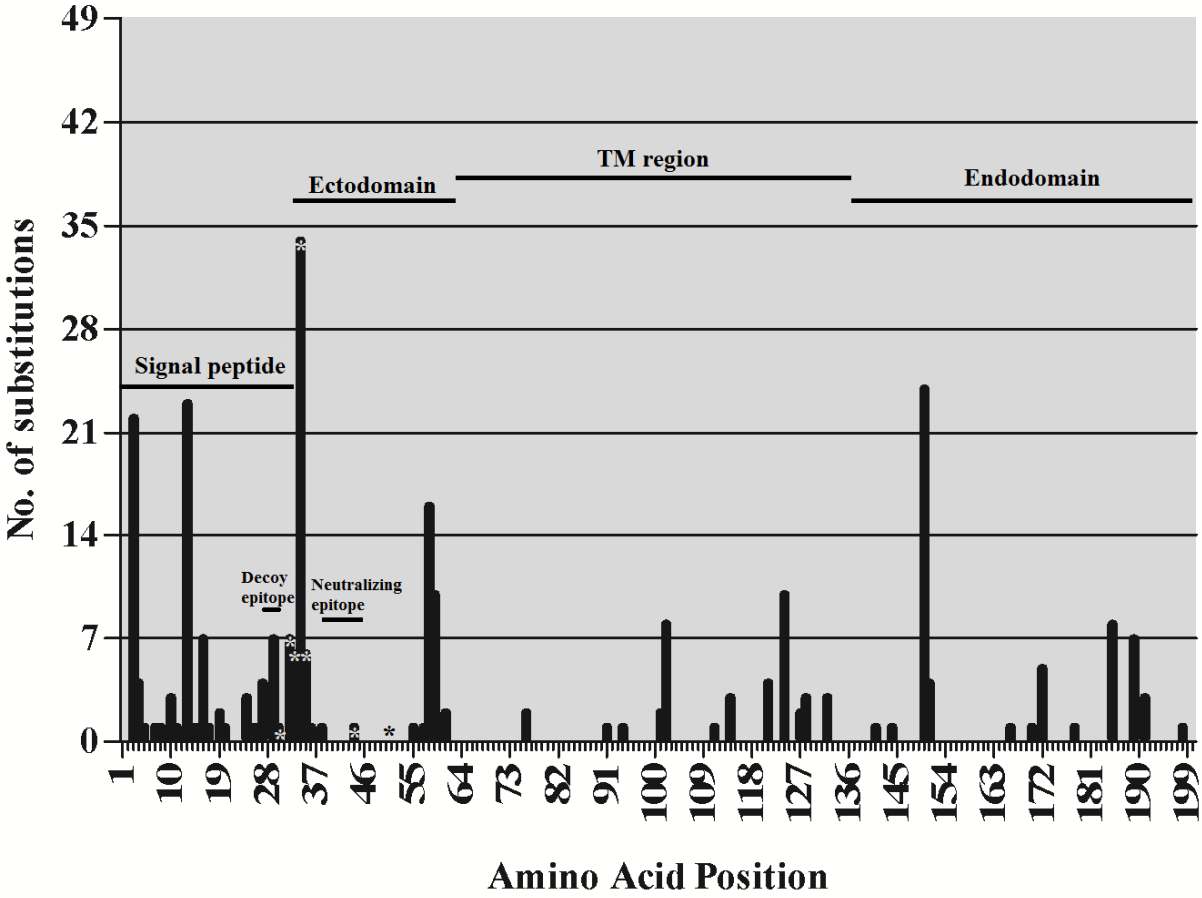

Fig. 3. Phylogenetic analysis of 11 Danish complete PRRSV Type 2 genomes and 10 complete genomes of viruses isolated in China and North America. The tree was constructed by the neighbor-joining method using CLC Main workbench (v. 6.6.2). Bootstrap values were calculated from 1,000 replicates and shown on branches. The scale bar represents 15% nucleotide change. PRRSV Type 1 Lelystad is used as outgroup (set root above node). CHN = China, DK = Denmark, US = United States of America.

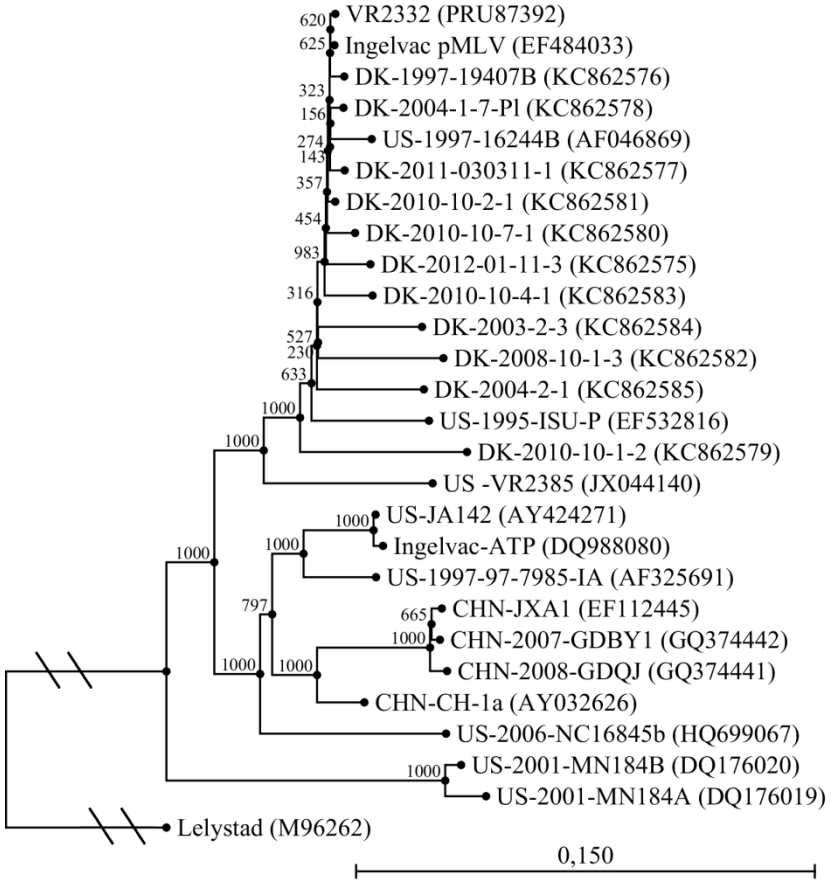

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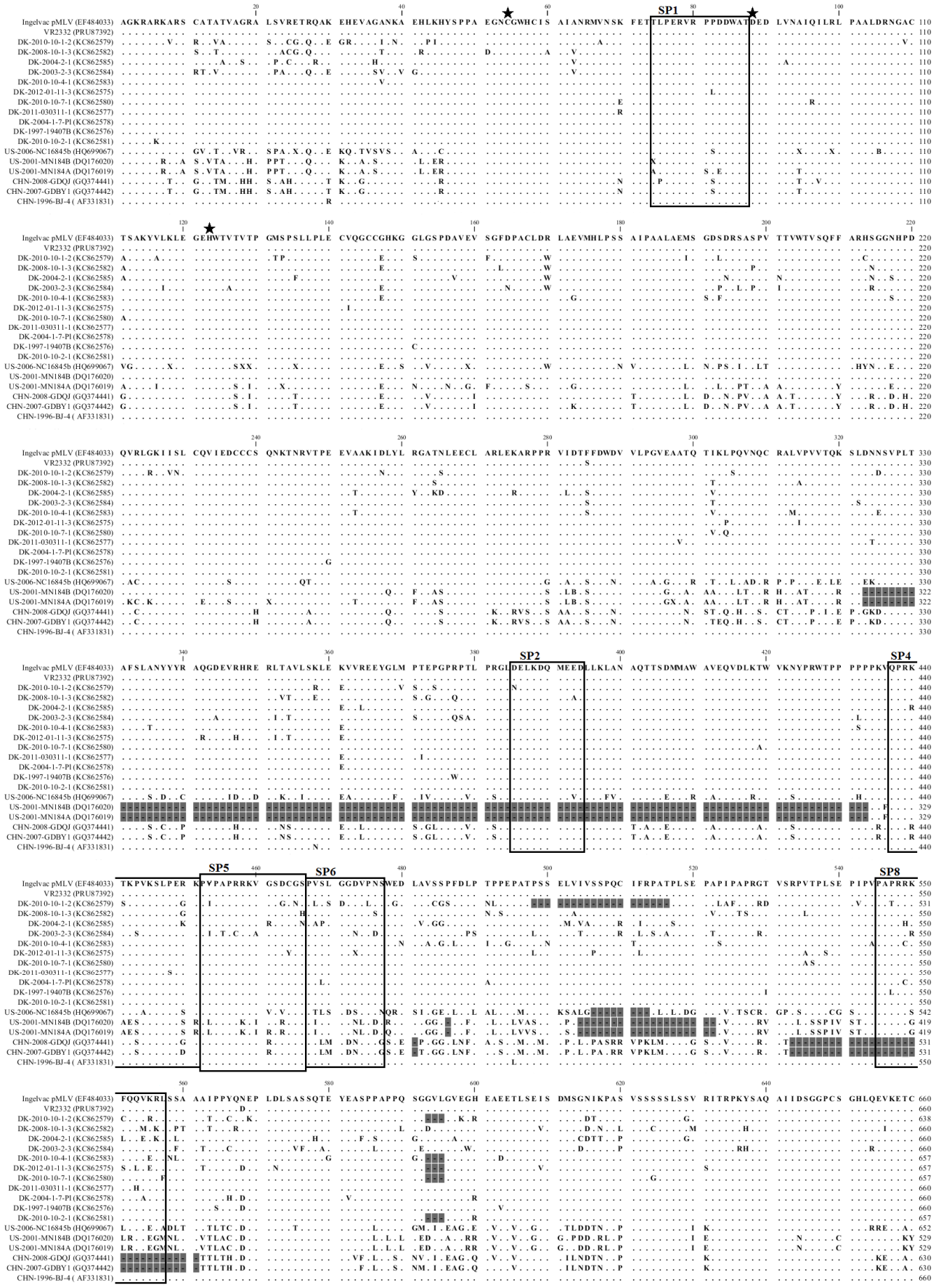
Fig. 4. Amino acid alignment of nsp2. Putative B-cell epitopes (Yan et. al 2007) are highlighted in black boxes. The amino acid residues forming the putative catalytic triad C54, D88, and H123 (Han et al., 2009) are marked with black stars. Deletion sites are marked with grey rectangles.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SP2
SP4
SP5
SP6
SP8

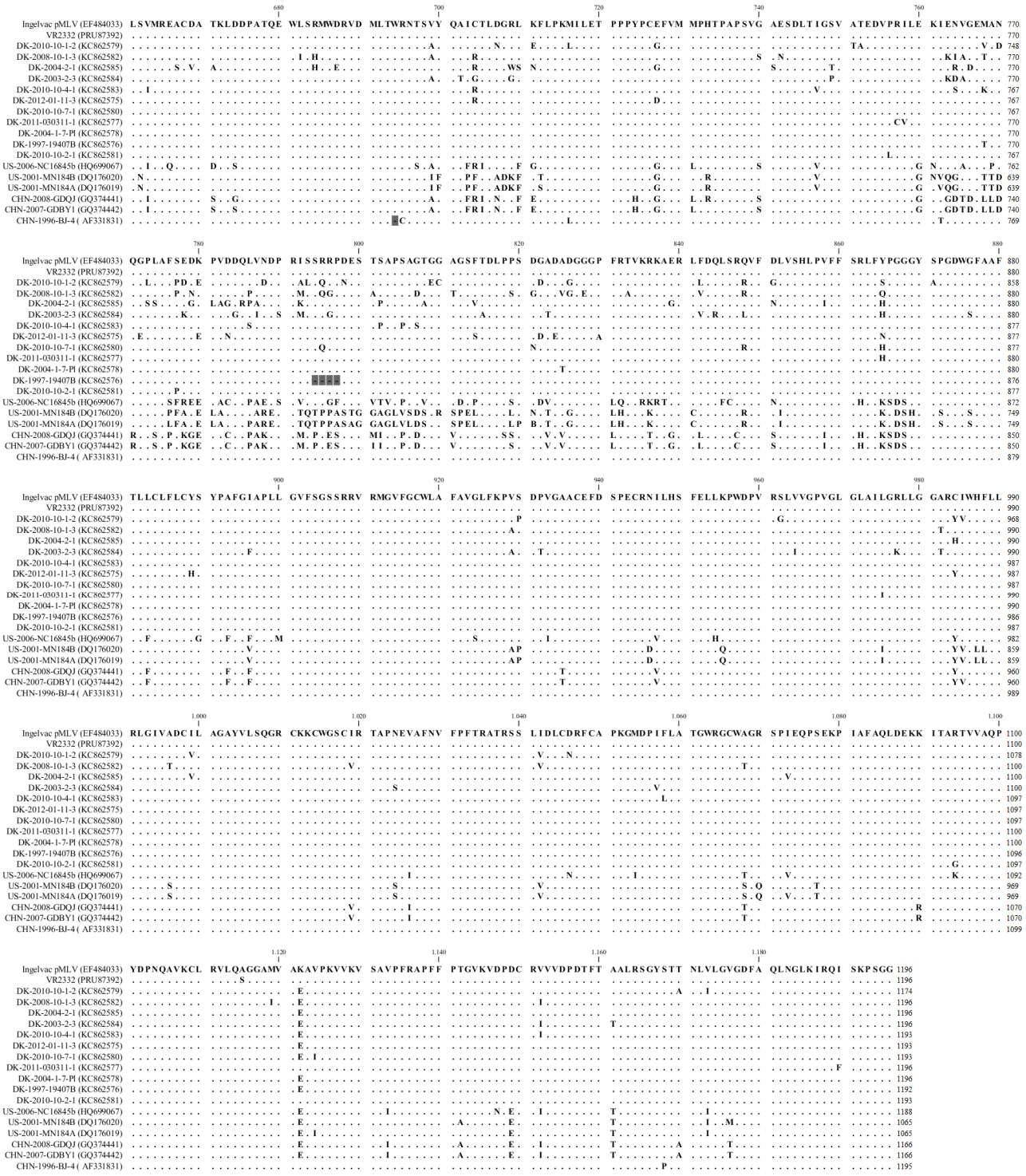


Fig. 5. Amino acid alignment of GP2. The 11 Danish GP2 amino acid sequences were aligned against the North American virus, US-1997-97-7895 used for detection of B-cell epitopes (de Lima et. al 2006). The two putative B-cell epitopes (No. 102 and 110) are highlighted with a black box. The putative N-glycosylations sites (N178 and N184) are marked with *.

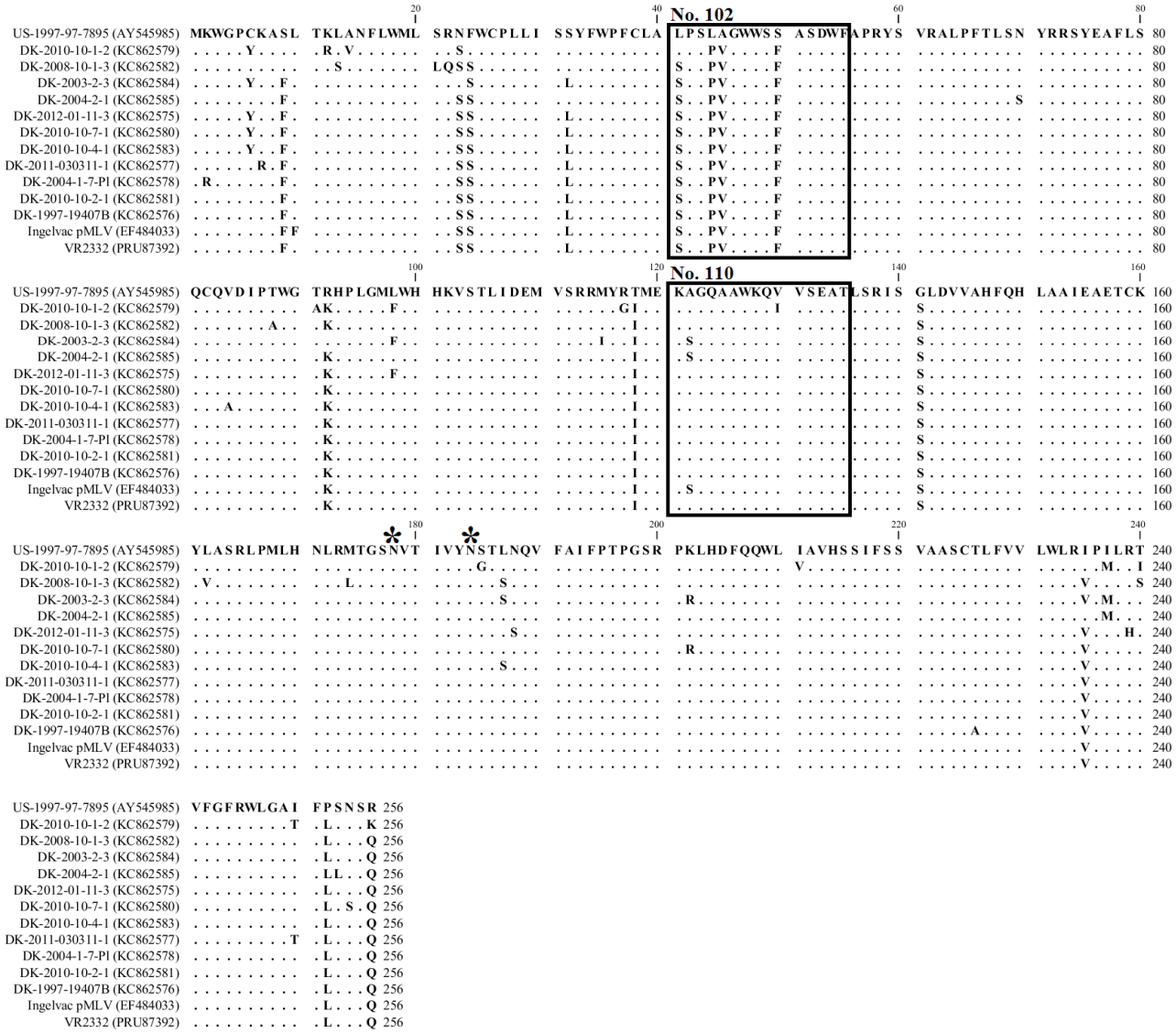


Fig. 6. Amino acid alignment of GP3. The 11 Danish GP3 amino acid sequences was aligned against the North American virus, US-1997-97-7895, used in detection of B-cell epitopes (de Lima et. al 2006). The four overlapping putative B-cell epitopes (no. 129-132) are highlighted with black boxes. The seven putative N-glycosylations sites (N29, N42, N50, N131, N152, N160, and N195) are marked with *.

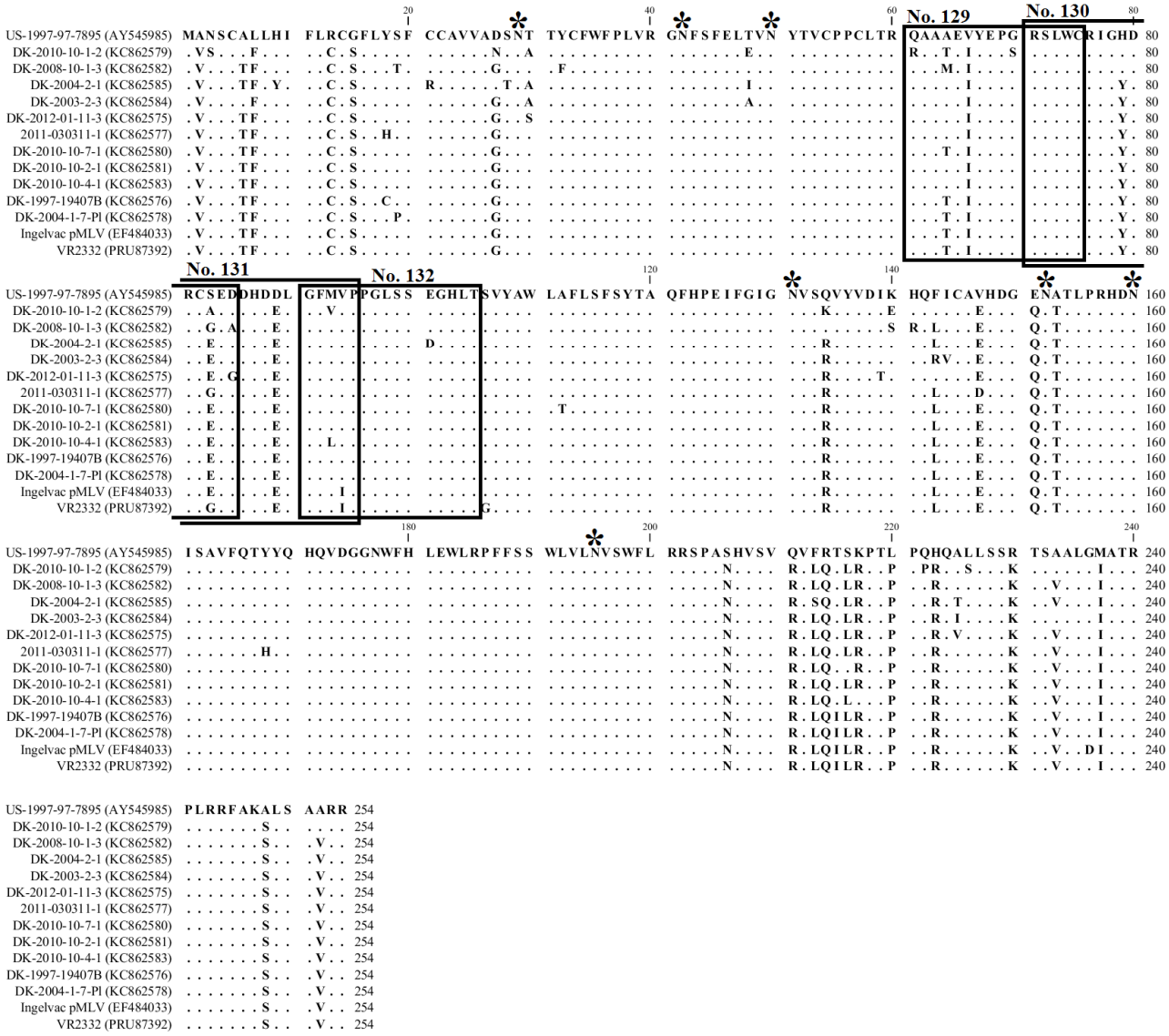
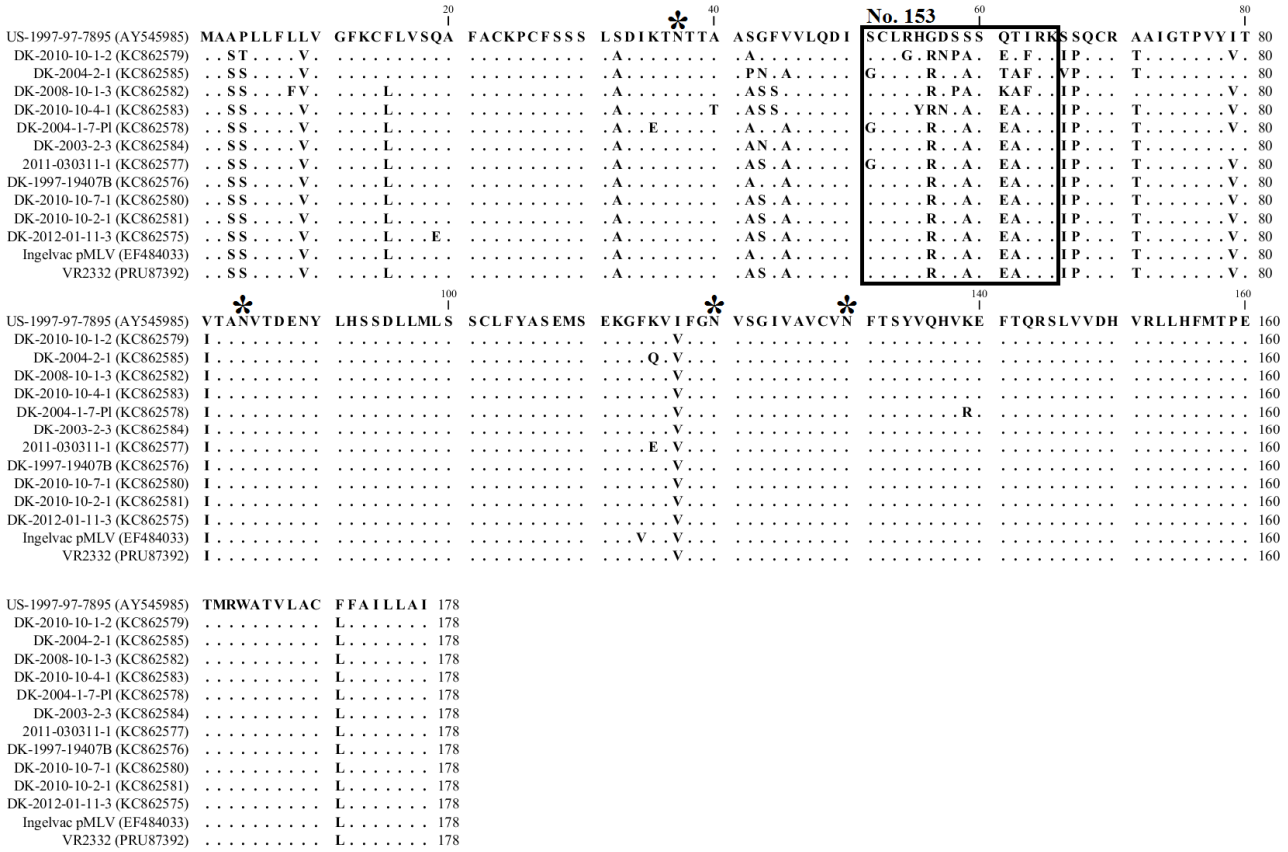


Fig. 7. Amino acid alignment of GP4. The 11 Danish GP4 amino acid sequences was aligned against the North American virus, US-1997-97-7895, used in detection of B-cell epitopes (de Lima et. al 2006). The one putative B-cell epitope (no. 153) is highlighted with a black box. The four putative N-glycosylations sites (N37, N84, N120; and N13) are marked with *.



Manuscript IV

Genetic and biological characterization of a Type 2 Porcine Reproductive and Respiratory Syndrome Virus causing significant clinical disease in the field

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(Prepared for submission)

Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the cause of significant reproductive and respiratory disease in swine worldwide. In Denmark approximately 50 % of the herds are seropositive for PRRSV of either or both genotypes.

In November 2010, a pig herd in the Northern part of Denmark experienced an infection with Type 2 PRRSV where the clinical impact appeared to be much more severe than normal reported from Danish Type 2 PRRSV affected herds. Due to the clinical observations of reproductive failures in sows and high mortality in piglets, it was speculated that a new, more pathogenic PRRSV strain had evolved in Denmark. The overall aim of the present study was to make a genetic and biological characterization of the virus isolated from the herd. Complete genome sequencing of isolates from the case herd revealed that although the case strain had some unique genetic features it was in overall very similar to the vaccine strain and to older Danish isolates. In an experimental trial, the virus induced no overt clinical signs in pigs following intranasal inoculation of the case strain or an older Danish strain. Virus shedding, acute phase responses and, serological responses were also comparable. Vaccination with a commercial MLV Type 2 vaccine had significant effect on virus shedding, together with magnitude and duration of viraemia. Combining the genetic and biological achieved data indicates that the severe disease seen in the field are influenced by other factors than the virus and emphasize that research aimed at identifying virulence maker of PRRSV are highly needed.

Keywords:

PRRSV, Type 2, Europe, Pigs, experimental infection

1. Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) belongs to the *Arteriviridae* family within the order *Nidovirales* (Cavanagh. 1997) and is the cause of significant respiratory and reproductive disease in swine worldwide (Han et al. 2006; Tian et al. 2007; Karniychuk et al. 2010). PRRSV is an enveloped single-stranded positive-sense RNA virus with a genome size of 15.0- 15.5 kb and contains a 5'-end cap and 3'-end polyadenylated tail (Meulenberg et al. 1998). The genome encodes at least 10 open reading frames (ORFs), including the recently discovered ORF5a (Meulenberg et al. 1993; Wu et al. 2001; Firth et al. 2011; Johnson et al. 2011). Based on nucleotide sequence comparison of early isolates of European and North American PRRSV it was found that they only shared 50-60 % nucleotide identity, hence PRRSV was divided into two major genotypes; Type 1 for the European isolated viruses and Type 2 for the North American isolated (Allende et al. 1999). Today both genotypes circulate worldwide (Shi et al. 2010). In Denmark, Type 1 PRRSV was first recognized in 1992, and in connection to an extensive vaccination program in 1996, Type 2 PRRSV was introduce (Botner et al. 1997; Madsen et al. 1998). Approximately 50 % of the Danish herds are seropositive for PRRSV antibodies against either or both types of PRRSV.

From time to time highly virulent PRRSV strains evolve, which results in impaired animal welfare and huge economical losses for the swine industry (Neumann et al. 2005; Han et al. 2006; Tian et al. 2007; Karniychuk et al. 2010). In 2006, China experienced the emergence of a highly pathogenic strain of PRRSV, which was similar to other Chinese Type 2 PRRSV strains in ORF5, but had indeed unique feature differences in other genes (Tian et al. 2007; An et al. 2010). The PRRSV epidemic in China affected more than 2 million pigs with about 400,000 fatal cases. This abrupt occurrence of high pathogenic PRRSV strains emphasizes the significance of monitoring the diversity of circulating PRRSV strains around the world both in respect to the sensitivity and specificity of diagnostic tests as well as efficacy of available vaccines.

In November 2010, a severe case of PRRSV with high mortality rate in piglets occurred in a farrow-to-finisher herd in the northern part of Denmark. By real-time RT-PCR, Type 2 PRRSV was detected in lung tissue samples from 10 days old piglets submitted to the National Veterinary Institute for routine diagnostics. The samples were negative for swine influenza virus, Porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae*. The infection was subsequently attempted to be controlled by extensive vaccination with Ingelvac® PRRS MLV and tightened of management procedures. From week 6 until week 14 after the initial infection, up to 75 % of the

fetuses were born as mummified. In February 2011 (12 weeks after the initial infection were recognized in piglets), pleura and lung tissue from mummified fetuses and stillborn piglets were found positive for PRRSV by real-time RT-PCR. The fetuses were negative for porcine parvovirus (PPV), PCV2 and Leptospira spp. Approximately15 weeks following the initial infection the number of liveborn piglets and the mortality until weaning was back to normal (33 weaned piglets per sow per year). Total losses of piglets until weaning for the 15-week period were about 30 %. The average loss in the nursery was 9.3 % until 18 weeks post the initial infection. Nineteen (19) weeks after the initial infection, post wean mortality were back to normal (2.2%).

Since the clinical impact appeared to be much more severe than normal reported from Danish Type 2 PRRSV affected herds, it was speculated that a new, more pathogenic PRRSV strain had evolved in Denmark similar to the strains that have previously evoked in Minnesota, China and Eastern Europe. However, the severity could also be due to a variety of other factors related to management and/or other infections. The overall aim of the present study was to make a genetic and biological characterization of the virus isolated from the herd by full-genome sequence analysis of the isolate and by comparing the infection dynamic of the virus isolated from the herd with an older Danish Type 2 isolate by experimental infection in young pigs. An additional aim was to assess the protective effect of a MLV Type 2 vaccine against this recent Danish Type 2 PRRSV isolate.

2. Materials and methods

2.1. Case samples

Samples from the case herd were collected in November 2010, February 2011 and March 2011. In November 2010, lung samples from nine 10-day-old piglets were obtained. In February 2011, seven (7) lungs from mummified fetuses and 13 lungs and 11 pleura samples from stillborn piglets were obtained from the same pig herd. In March 2011, lungs and livers from three stillborn piglets and lungs and/or hearts and livers from 11 mummified fetuses were obtained.

From the samples submitted in November 2010, one virus, designated DK-2010-10-13-1, was isolated in Marc-145 cells using general cell culture procedure (Kim et al. 1993).

2.2. RNA extraction from field samples

Lung tissue was initially prepared as a 5 % homogenate in RLT buffer (QIAGEN) containing 1 % β-mercaptoethanol (Sigma-Aldrich). RNA was extracted from lung homogenate and pleura using

RNeasy Minikit (QIAGEN) according to the manufacturer's instructions. Total RNA from cell culture supernatant for full genome sequencing was purified using QIAamp Viral RNA Mini Kit (QIAGEN). The elution volume for both extractions methods was 60 µl. The RNA was stored at -80°C until use.

2.3. Real-time RT-PCR

The extracted RNA was screened for PRRSV using a modification of the Primer Probe Energy Transfer RT-PCR (PriProET-RT-PCR) assay described by Balka, 2009 (Balka et al. 2009).

2.4. cDNA and conventional PCR

Full-genome cDNA synthesis was performed by SuperScript® III First-Strand Synthesis System (Invitrogen) as described elsewhere (Kvisgaard et al. 2013b). PCR amplification of partial-NSP2 (Zhou et al. 2009), ORF5 and ORF7 (Oleksiewicz et al. 1998) was carried out using the AccuPrime™ *Taq* DNA Polymerase High Fidelity Kit (Invitrogen). The PCR mixture was prepared as recommended by the supplier except that the amount of AccuPrime™ Taq High Fidelity was increased to 0.5 µl and 2 µl cDNA was used as template. The PCR amplification was conducted on a T3 thermo cycler (Biometra) with the following conditions: [94°C for 15 seconds], 45 cycles: [94°C 15 seconds, 55°C for 30 seconds, 68°C for 60 seconds] then finalize with 68°C for 5 minutes and cool down to 4°C. PCR primers for amplifying ORF5 and ORF7 were from Oleksiewicz, 1998 (Oleksiewicz et al. 1998).

Long range PCR amplification was performed using the full-genome cDNA as template with AccuPrime™ Taq High Fidelity kit as described by Kvisgaard et al. 2013.

2.5. Cycle sequencing and next generation sequencing

PCR products of partial-NSP2, ORF5 and ORF7 were sequenced by cycling sequencing using the Sanger method using the PCR primers as sequencing primers (Sanger et al. 1977). The complete genome of DK-2010-10-13-1 was obtained using the 454 GS Jr sequencer from the laboratory of Fred Leung, The University of Hong Kong, Hong Kong, China. DNA templates for the isolate was generated using a random PCR method previously described (Van Doorsselaere et al. 2011). The complete genome of DK-2011-88005-A8-Pl was obtained using the Ion Torrent GPM sequencer (DTU Multi-Assay Core (MAX), Kgs. Lyngby, Denmark) as described elsewhere (Kvisgaard et al. 2013).

2.6. Data analysis

Data analysis of sequences obtained from cycle sequencing was carried out using the commercial software CLC Main Workbench v. 6.6.2 (CLC BIO, Arhus, Denmark). Contigs of ORF5 and ORF7 were produced from assembling the raw data obtained for cycle sequencing against the reference sequence VR2332 (PRU87392). The complete genome of DK-2011-88005-A8-Pl was achieved by the mapping of reads using the Burrows-Wheeler aligner (BWA) (Kvisgaard et al. 2013b). Amino acid sequences were predicted from the nucleotide sequences using CLC Main Workbench v. 6.6.2. Nucleotide and amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log- Expectation).

2.7. Inoculation material for experimental infection

The viruses were propagated in Marc-145 cells using the general cell culture procedure (Kim et al. 1993). For both viruses, a second passage of cell culture supernatant was used as the inoculation material. The DK-1997-19407B isolate, an older Danish PRRS Type 2 virus, had a titer of $5.4\log_{10}$ TCID50/ ml. The DK-2010-10-13-1 isolate had a titer of $5.9\log_{10}$ TCID50/ ml. One milliliter of cell culture supernatant was diluted to a total volume of 4 ml and administered intranasally to each pig with 2 ml per nostril.

2.8. Experimental set-up

Twenty-eight 4-week-old pigs (Landrace X Yorkshire X Duroc cross-breeds) were purchased from a commercial pig production herd seropositive for Actinobacillus pleuropneumoniae (AP) serotypes 6 and 12, but declared free from a range of important swine pathogens including Mycoplasma hyopneumoniae, PRRSV and AP serotypes 1, 2, 3, 4, 5, 7, 8, 9 and 10 according to the Danish Specific Pathogen Free (SPF) system. The pigs were randomly divided into one control group with four pigs (group 1) and three experimental groups (groups 2-4) each with 8 pigs. The individual groups were housed in separate sections of the BSL3 animal isolation facilities at the National Veterinary Institute, Lindholm. After an acclimatization period of 5 days, the pigs in group 4 (pig no. 21 – 28) were vaccinated (2 ml intramuscularly (i.m)) with the Ingelvac® PRRS MLV vaccine (Boehringer Ingelheim Animal Health; Ingelheim, Germany). Four weeks later, at post inoculation day 0 (DPI 0), the pigs in group 1 (pig no. 1-4) were sham- inoculated intranasally (i.n.) with Eagle's MEM. The pigs in group 2 (pig no. 5-12) and group 3 (pig no. 13-20) were inoculated i.n. with the DK-1997-19407B and the case DK-2010-10-13-1 isolate, respectively. Pigs in group 4

(pig no. 21 -28) were challenged with the DK-2010-10-13-1 case isolate. The group 1 and group 4 pigs were euthanized on DPI 29 and the other two groups on DPI 30. Euthanasia was performed by intravenous injection of pentobarbiturate (50 mg/kg) followed by exsanguination by cutting the *arteria axillaris.* The study was carried out in accordance with the Danish legislation on animal experiments (LBK nr 1306 – 23/11/2007) and EU regulations on the use of laboratory animals for research.

2.9. Clinical examination

Individual pigs were subjected to daily clinical examination and body rectal temperatures were recorded. To avoid influence of handling on the body temperature recordings, these were usually carried out while pigs were eating. On a few occasions, restrainment of pigs was necessary to measure the temperatures. In order to obtain a semi-quantitative measure for comparison of clinical disease between the 4 groups, all pigs were scored using the clinical scoring (CS) system developed for swine fever infections by Mittelholzer et al. (2000) and modified for PRRSV experiments focusing on PRRS typical clinical signs in growing pigs, - evaluating parameters like common well-being, appetite, respiration, and eye-disorders.

2.10. Sampling

Blood samples were collected from all pigs on post inoculation days (DPI) -28, 0, 3, 7, 10, 14, 21, 28, and 29/30 (days of euthanasia) for clinical, virological, haematological and immunological examination. Samples obtained on DPI -28 and 0 were collected prior to vaccination and inoculation, respectively. Blood samples were collected in 5 or 10 ml vacutainers (Venoject; Terumo Europe, Leuven, Belgium) from the anterior vena cava. Non-stabilized whole-blood samples were left on ice to coagulate for 15 minutes and centrifuged at 3500 rpm for 10 minutes at 4°C. Serum was separated and stored at -80°C for subsequent analysis. In parallel to the blood samplings, nasal swab samples were collected on the same days except on day -28, 29/30 PI from the right nostril. The swabs were placed in 1 ml PBS (pH 7.5) and stored at -80°C until further analysis.

2.11. Tissue sampling

At post mortem, two identical samples from the following tissues were collected: nasal mucous membrane, tonsil, trachea (the middle part), tracheobronchial lymph node, liver, spleen, and lung

tissue from the right apical- and diaphragmatic lobes, the intermediate lobe and from lesions, if present. One of each sample was fixed in 10% neutral buffered formalin. The other tissue samples were stored at -80°C.

2.12. Total WBC counts

Total WBC counts were carried out on freshly collected EDTA-stabilised blood samples using a semi-automated animal blood cell counter (Vet abc^{TM}, ABX, Montpellier, France). All samples were counted twice, and the mean value was used.

2.13. Flow cytometry analyses

Phenotyping of leukocytes in peripheral blood was carried out by flow cytometry, using single as well as triple labeling methods to define different sub-populations. Single-colour analysis was applied to differentiate leukocyte populations and for B-cell detection, as previously described (Nielsen et al. 2003b). Phenotyping of peripheral blood T-cell subpopulations was performed by triple-color flow cytometry using CD3/CD8/CD4 labelling, as previously described (Nielsen et al. 2003b). Selection of gates was as described elsewhere (Summerfield et al. 2001). This permitted the identification of the T-cell subpopulations as $CD3^+CD4^+CD8^-$ naïve Th cells, $CD3^+CD4^+CD8^+$ as memory/activated Th cells, $CD3^+CD4^-CD8^+$ as Tc cells, $CD3^+CD4^-CD8^{low//-}$ as γδ T cells. NK cells were defined as $CD3^-CD4^-CD8^+$ cells.

2.14. Quantification of viral load in experimentally infected pigs

Total RNA was extracted from 100 µl serum and nasal swab samples with QIAsymphony® SP robot using QIAsymphony® RNA Kit with protocol RNA CT 400 v.5 and elution volume 100 µl according to instructions from the supplier (QIAGEN). Known PRRSV positive samples and negative controls were included in each 24-sample batch of RNA purifications. Extracted RNA was stored at -80˚C.

For real-time RT-PCR, the previously published "Kleiboeker mod-1" primers and probe targeting Type 2 PRRSV ORF7, 3'UTR (Wernike et al. 2012) were used. The PCR mix contained 2 µl template RNA, or nuclease free water (Amresco, Medinova, Denmark) for no template controls together with 10 µl nuclease free water (Amresco, Medinova, Denmark), 5 µl 5x QIAGEN OneStep RT-PCR buffer (QIAGEN®, Cat. No. 210215), 1 µl dNTP mix [10 mM each], 0.75 µl primer NAfw 5'-ATRATGRGCTGGCATTC-3' [10 pmol/µl], 0.75 µl primer NArev 5'-

ACACGGTCGCCCTAATTG-3', 0.5 µl dual labeled probe 5'-TEX-TGTGGTGAATGGCACTGATTGACA-BHQ2 [10 pmol/µl] and 1 µl QIAGEN OneStep RT-PCR Enzyme Mix. Amplifications were performed on RotorgeneQ (QIAGEN®) with temperature profile: 30 min at 50˚C for reverse transcription followed by 15 min at 95˚C, and 45 cycles of 15 sec at 94˚C, 60 sec at 60˚C and 10 sec of 72˚C. Fluorescence signals were collected during the extension step of each cycle in the Yellow channel and analyzed with Rotor-Gene Q software version 2.0.2 (QIAGEN) setting NTC threshold at 10 % and the normalised fluorescence threshold limit at 0.01 for cycle threshold (Ct) value determination starting normalising from cycle 2. Samples were tested in duplicates, and determined positive when both replicates were positive with a Ct less than 37.

Quantifications of viral load in experimental samples were performed against standard curves matching each of the inoculation virus isolates and imported into each RT-PCR run with adjustment according to a PRRSV RNA calibrator sample included in triplicate in each real-time RT-PCR run.

Standard curves for quantification of DK-2010-10-13-1 virus were obtained using a five-fold dilution series of 5.9 log10 TCID50/ml of DK-2010-10-13-1 inoculum virus isolate spiked 1:100 into PRRSV negative swine serum and nasal swab material, respectively. RNA was extracted from each spiked dilution and tested in triplicate by real-time RT-PCR in at least two independent PCR experiments. The serum and nasal swab standard curves had respectively a PCR efficiency of 97 % (R^2 = 0.99, slope = -3.398) and 91 % (R^2 = 0.99; slope = -3.547) and their range of quantification covered 7 and 6 log5 dilution steps corresponding to concentrations 0.5 - 8.0E+03 and 0.52 – 8.1E+03 TCID50/ml equivalents. Similarly, standard curves for quantification of DK-1997-19407B virus in serum and nasal swabs were produced by spiking of 5.4 log10 TCID50/ml DK-1997-19407B inoculum virus isolate in PRRSV negative serum and nasal swab material. They had PCR efficiencies 98 % (R^2 = 0.98; slope = -3.374) and 93 % (R^2 = 0.99; slope = -3.909), respectively, and both covered 6 log5 dilution steps corresponding to concentrations 0.5 - 1.4E+03 and 0.14 – 2.3E+03 TCID50/ml equivalents, respectively.

2.15. Serology

Antibodies in sera from experimentally infected pigs were analyzed using a PRRSV genotype discriminating IPMA (Sorensen et al. 1997) and a genotype discriminating blocking ELISA (Sorensen et al. 1998). For IPMA, the serum was initially diluted 1:50 and then tested using a fivefold dilution series (1:50-1:6250). The results were expressed as the highest dilution generating

a positive signal (titer). In ELISA, the serum were tested diluted 1:2 and the results expressed as blocking percentage (OD %). A sample was considered positive if the OD percentage was below 44.

2.16. Acute phase proteins

Plasma levels of haptoglobin was determined by a sandwich ELISA using an in-house mouse anti-porcine haptoglobin monoclonal antibody as catching antibody and a commercial rabbit anti-human haptoglobin detection antibody (DAKO A0030, DAKO, Glostrup, Denmark) as previously described [REF1] . CRP was analyzed by a sandwich type ELISA using dendrimer-coupled cytidine diphosphocholine (a CRP-binding ligand) in the coating layer as described in [REF4] employing polyclonal rabbit anti-human antibodies with cross-reactivity towards porcine CRP followed by peroxidase-conjugated goat anti rabbit antibody for detection (both antibodies from DAKO, Glostrup, Denmark).

2.17. Statistics

Unpaired t-tests with 95 % confidence interval were performed for the comparisons of responses between groups using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

3.1. Screening of case samples for PRRSV by real-time RT-PCR

All nine lungs from 10 days old piglets collected in connection to the initial clinical signs in November 2010 were found positive for Type 2 PRRSV by real-time RT-PCR screening. Two lungs from mummified fetuses and three lungs and one pleura sample from stillborn piglets in February 2011were positive for Type 2 PRRSV by real-time RT-PCR. One lung from stillborn piglets and 9 lungs from mummified fetuses collected in March 2011 were also positive for PRRSV Type 2.

3.2. Genetic and antigenic analysis of NSP2 (partial), ORF5, and ORF7

ORF5 and ORF7 were sequenced from all nine lungs obtained from the 10 days old piglets in November 2010. These case viruses were almost (>99.2%) identical both genes. The pairwise

nucleotide sequence identity between ORF5 and ORF7 of these viruses to Ingelvac PRRS MLV was 97.2-97.4 % and 98.6 %, respectively. Alignment of the deduced amino acid sequence of ORF5 revealed some amino acid variations in the glycoprotein 5 (GP5) of the case viruses compared to the vaccine strain and other Danish PRRSV Type 2 viruses (fig. 1). Some of the variations in GP5 of the case viruses were also present in the high pathogenic (HP) PRRSV strains from China. The HP-PRRSV strains from China had also showed to harbor a discontinuous deletion of 1 plus 29 amino acids in the non-structural protein 2 (nsp2), encoded in ORF1a of the PRRSV genome (Tian et al. 2007). The variations in GP5 of the case viruses led to the sequencing of a partial part of the coding region of NSP2 including the region where the Chinese HP-PRRSV strain had deletions. The amino acid alignment of partial nsp2 encoded by the case viruses and three Chinese HP-PRRSVs are shown in fig. 2. The case viruses did not harbor the same deletions as the HP-PRRSV strains, but instead a 3 amino acid deletion was detected. Blast searches did not reveal any published viruses harbouring this 3 amino acid deletion in nsp2 of Type 2 PRRSV. Besides the 3 amino acid deletion in nsp2 several amino acid variations were detected compared to the vaccine strain (fig. 2). The pairwise nucleotide sequence similarity of partial NSP2 to the vaccine strain was 94.3-95.0 %.

Comparison of ORF5, ORF7, and partial NSP2 sequences obtained from viruses from the initial infection in November 2010 to the sequences obtained from mummified fetuses and stillborn piglets approximately 12 weeks later confirmed that it was the same virus strain still circulating in the pig herd. Thus, the pairwise nucleotide sequence identities between the two viruses were for ORF5, ORF7, and partial NSP2 99.3-99.8 %, 99.7-100 %, and 99.2-100 %, respectively. The amino acid alignments shown in fig. 1 and fig. 2 further confirmed the similarity of these viruses with 98.5-100 %, 100 %, and 98.8-100 % pairwise amino acid identities in GP5, N, and partial nsp2 respectively.

3.3. Complete genome sequence comparison

Complete genome sequences were obtained from PRRSV extracted from lung tissue and pleura submitted in November 2010 (DK-2010-10-13-1) and February 2011 (DK-2011-88005-A8-Pl). The complete genomes were 15,402 nucleotides long (excluding the poly(A)-tail) and the pairwise nucleotide identity between the two genomes was 99.7 %. The identity to the vaccine strain was 97.8 - 97.9 %. Apart from the 9 nucleotides in NSP2 no further deletions was observed.

3.4. Clinical signs and body temperature

Generally, all pigs remained healthy throughout the experimental period, and only very few deviations from normal health was seen (data not shown). Pig no 7, which was slightly lethargic, had increased respiratory rate at eating and showed increased body temperature (40.2°C) on DPI 18, only. Short (1-3 days) episodes with semi-liquid feces were observed occasionally in all groups. Increased body temperatures (fig. 3) could be related to restraint of pigs or to the episodes with semi-liquid feces for some of the pigs in group 4.

3.5. Gross pathology

At necropsy, no lesions were seen in the control pigs (no. 1-4) and only minor lesions were revealed among the remaining pigs.

In group 2, three pigs (nos. 5, 6, 8) were without lesions. Observed lesions were focal lobular condensation in the cranial lung lobe (no. 9) and mild enlargement of the tracheobronchial lymph nodes (nos. 7, 9, 10, 11). Pig no. 7 was pale and had chronic adhesive pleuritis and pericarditis. In three pigs (nos. 7, 10, 12), the spleen was mottled and/or the margins of the spleen were slightly depleted.

In group 3, pigs nos. 15, 19 and 20 had no lesions. Findings were mild enlargement of the tracheobronchial lymph nodes (nos. 13, 14) and a mottled spleen with slightly depleted margins (nos. 16, 17). Pig no. 18 had a renal cyst.

Two of the vaccinated pigs (group 4) were without lesions (nos. 22 and 26). Findings in the cranial lung lobes were focal- (no. 21) or multifocal (no. 25) areas of condensation, and in the dorsal lung lobes ecchymotic hemorrhages (no. 24) and areas of collapsed lung tissue (atelectasis) (no. 27) was seen. In two pigs the margins of the spleen were slightly depleted (no. 23, 28).

3.6. Total WBC counts and blood leukocyte phenotyping

Total WBC counts were decreased at DPI 3 in pigs in groups 2 and 3 (fig. 4). This decrease was reflected by a reduction in absolute lymphocyte counts, caused by significantly reduced levels of B cells in both groups together with decreased numbers of CD8+ cells (T_C and NK cells) in group 2 pigs. At DPI 14, all values have returned to levels approximately corresponding to DPI 0 levels. For the remaining blood leukocyte subtypes, no significant differences were observed among the groups (data not shown).

3. 7. Real time RT-PCR on PRRSV

PRRSV was detected by real-time RT-PCR in all pigs inoculated with virus (groups 2-4). The viremia peaked at DPI 7 (fig. 5), but the viral load was significantly lower and of much shorter duration in the vaccinated group (gr. 4) compared to the unvaccinated animals ($p= 0.04$). In groups 2 and 3, the mean viral load reached approximately 3 log10 TCID50 equivalents and PRRSV remained detectable until the end of the experiment at DPI 28. The mean viral load was, however, higher the group of pigs inoculated with DK-2010-10-13-1 than with DK-1997-19407B at DPI 10 and 14 although not statistically different. Low levels of PRRSV were detected at DPI 0 in serum from 4 out of the 8 pigs in the vaccinated group and these results were reproduced from a second RNA extraction from the same serum samples. Contrary, none of the pigs in the other groups were positive by real-time RT-PCR at DPI 0.

Virus excretion in nasal swabs peaked with approximately 1 log10 TCID50/ml equivalents at DPI 7 for the virus inoculated and unvaccinated groups of pigs and rapidly declined (fig. 6). Virus was detected in nasal swab samples from all pigs in the virus inoculated non-vaccinated groups starting at DPI 3. In the group of pigs inoculated with DK-1997-19407B, virus was detected until DPI 7 in 3 pigs, until DPI 10 in 4 pigs, and until DPI 14 in one pig. The DK-2010-10-13-1 inoculated pigs were positive for virus in their nasal swab samples until DPI 10 for 6 pigs, one pig was positive until DPI 14 and the last pig in that group only had detectable levels of virus at DPI 21. In the vaccinated group, only two out of the eight pigs showed detectable levels of virus in nasal swabs, and only at DPI 10.

3.8. Serology

In ELISA, all animals were clearly negative at day -28 and only vaccinated animals (group 4) were positive at DPI 0 (fig. 7). Following the inoculation at DPI 0, all animals in groups 2-4 seroconverted at DPI 7 followed by a steady increase in level of antibodies that remained high until the end of the study at DPI 28. A similar development in IPMA titers were observed (fig. 8), however the animals in group 2-4 seroconverted at DPI 14 in IPMA compared to DPI 7 in ELISA.

3.9. Acute phase protein measurements

The acute phase responses were included as an objective measure of the severity of infection. Results from measurements of the acute phase proteins C - Reactive Protein (CRP), and haptoglobin are shown in fig. 9 and 10, respectively. The unvaccinated and PRRSV inoculated

groups of pigs (groups 2-3) showed a clear CRP response following inoculations at DPI 0, peaking on DPIs 7 and with a second peak at DPI 21 (group 2 only). Similarly, haptoglobin peaked at around DPI 10 in both groups. The CRP and haptoglobin responses of the pigs in the vaccinated group were similar to the responses of the pigs in the sham inoculated control group and showed some random increases and decreases throughout the observations period.

There was a significant difference in haptoglobin concentration between group 1 (controls) and group 2 when using an unpaired t-test with 95 % confidence interval ($p=0.04$) (fig. 10). No other significant differences were seen between the groups.

4. Discussion

The aim of the study was to perform a genetic characterisation of a PRRS virus isolated from a herd with severe reproductive and respiratory symptoms and to compare the "virulence" of the virus with an older Danish Type 2 isolate *in vivo*. Although the case strain DK-2010-10-13-1 had some unique genetic features it was overall very similar to the vaccine strain and to older Danish Type 2 isolates. Furthermore, the virus induced no overt clinical signs in pigs following intranasal inoculation.

Terms such as "high and low pathogenicity", "high and low virulence", and "hot strains" are often interchangeable used to allocate PRRSV isolates into pathotypes, but efforts to link specific genetic motifs to different biological and immunological features has so far been unsuccessful. Thus, until now no clearly defined molecular marker of virulence have been established for PRRSV (Opriessnig et al. 2002; Wu et al. 2009) . The 1 + 29 amino acid deletions seen in the nsp2 of High pathogenic PRRSV stains in China were at first believed to be a virulence marker, however, experimental infections performed with chimeric infectious clones and the finding of a low pathogenic field virus also harboring deletion at the same positions has subsequently shown that this unique discontinuous deletion was not directly related to virulence (Zhou et al. 2009; Li et al. 2010). The nucleotide sequences revealed that the DK-2010-10-13-1 strain was 96.9-97.4 % and 94.3-95.0 % identical to the vaccine strain in ORF5 and partial NSP2, respectively. The pairwise nucleotide identity between the sequences obtained from the viruses from the initial infection and those obtained approximately 12 weeks after were 99.3-99.8 %, 99.7-100 %, and 99.2-100 % identical in ORF5, ORF7, and partial NSP2, respectively. Taken together, these results documented that it was the same strain that was isolated 3 months apart, that this strain was different from the vaccine strain and that the strain belonged to the same group of Type 2 PRRS viruses found

elsewhere in Denmark (Kvisgaard et al. 2013a). The DK-2010-10-13-1 strain had a deletion of three amino acids in nsp2 not previously described for any Type 2 strains (Kvisgaard et al. 2013a). This unique 3 amino acid deletion was also present in viruses collected from 6 of 14 Danish pig herds (accession no. KF311033-57) (unpublished data). Five of these where isolated in 2010 and 2012 and one was collected in 2003. It is not known if these herds experienced very severe clinical signs so the use of this 3 amino acid deletion as a predictor/marker of virulence is not sustained by the available data.

Results of previous studies supported the concept that the magnitude and duration of viraemia is the best indicator of the "virulence" of a given PRRSV strain, however, a direct quantitative relationship between virulence and viral load has not been established (Haynes et al. 1997; Johnson et al. 2004; Weesendorp et al. 2013). In the present study, the level of virus were higher from DPI 3 and onwards in pigs infected with the DK-2010-10-13-1 virus in comparison to the older Danish DK-1997-19407B strain, however, the differences were not significant. Furthermore, the viraemia were of shorter duration and lees pronounced than described for other strains of PRRSV causing significant clinical disease and mortality in experimental trials (Johnson et al. 2004; Weesendorp et al. 2013). Thus, it is doubtful if these differences in duration and magnitude of viraemia between the case strain and an older Danish Type 2 strain can explain the severe clinical signs and mortalities seen in the herd from which this strain was isolated. On the contrary, the more pronounced reduction of B and CD8+ cells in animals inoculated with the older Danish isolate, indicated that the older Danish strain were more virulent than the case strain.

Neither overt clinical signs nor mortalities were seen in the present study albeit the pigs were indeed infected as documented by the significant acute phase protein responses and the development of antibodies against PRRSV. There are many possible explanations for differences in clinical impact in the field and in experimental set up`s. The virus used for the experimental infection was initially recovered from a foetus thus it is possible that more severe signs could evolve if a transplacental infection model had been used. Furthermore, others have previously failed to provoke severe clinical disease under experimental conditions with PRRSV isolated from severely affected herds (Halbur et al. 1996) probably because the clinical impact are influenced by other factors in the herd such as co-infections with swine influenza virus, PCV2, Mycoplasma and secondary bacterial infections (Harms et al. 2001; Schmitt et al. 2001). Indeed, the temporary decrease of total lymphocytes and B cell numbers in both unvaccinated groups of pigs indicated a

state of immunosuppressant following infection which may prone the animals for secondary infections.

The unchanged blood parameters and the lack of acute phase responses observed after infection of the vaccinated pigs indicated a protective effect of the vaccine against infection with the case strain despite the nucleotide difference between the vaccine isolate and the case strain was ranged from 3 to almost 6 % in some genes. Indeed, the viral load in serum was significantly higher and of longer duration in the groups of non-vaccinated virus inoculated pigs than in the group of vaccinated pigs. Similarly, the vaccinated group of pigs had significantly reduced virus excretion in nasal swabs, and only 2 out of 8 pigs had virus at detectable albeit low levels of virus at DPI 10, the remaining pigs were negative throughout the experiment. These results documented that vaccination with the Ingelvac® PRRS MLV vaccine (Boehringer Ingelheim Animal Health; Ingelheim, Germany) reduced viremia and excretion of the virus, thus being effective of limiting the impact of the DK-2010-10-13-1 case virus and would be expected to be effective in the control of an outbreak with this and related viruses. These findings are in accordance with the finding that the vaccine was used effectively in the control of the infected herd but contradicts the perception that a variability of more than 2% among field strain and vaccine strains results impair the of efficacy of the vaccine.

In conclusion, under the condition of the present study, a PRRS virus isolated from a herd experiencing very severe clinical signs and significant mortalities, failed to induce comparable signs in an experimental set-up. Full genome sequencing of the isolate revealed that the isolate was very similar to other Danish Type 2 isolates. Combining these data indicate that the severe disease seen in the field are influenced by other factors than the virus and emphasize that research aimed at identifying virulence maker of PRRSV are highly needed.

5. Acknowledgements

The assistance of the technical staff from the infection immunology laboratory and the endemic virology laboratory at Lindholm is appreciated. Likewise, we thank the animal care unit staff at Lindholm for handling the animals.

The genetic and biological examinations were funded from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 245141 (New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia

(PoRRSCon) coordinated by Prof. H. Nauwynck) and the experimental infection in pigs was kindly funded by Boehringer Ingelheim Animal Health, Germany.

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Fig. 1. Amino acid alignment of GP5

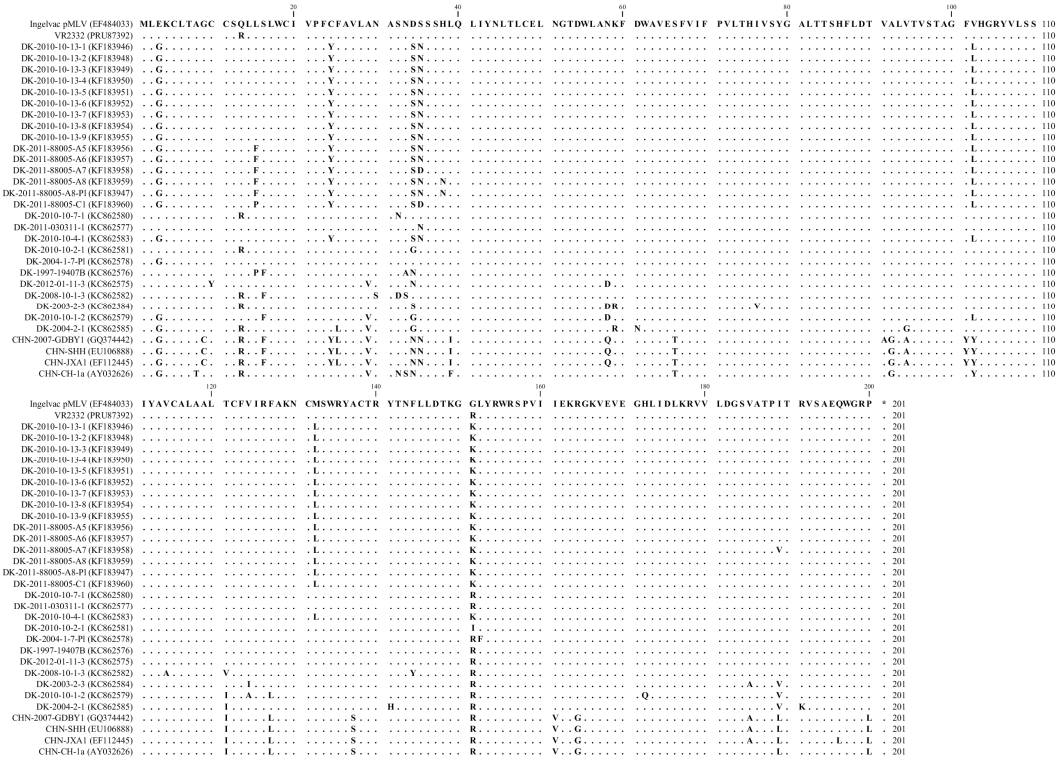


Fig. 2. Amino acid alignment of partial nsp2. Gray squares represents deletions

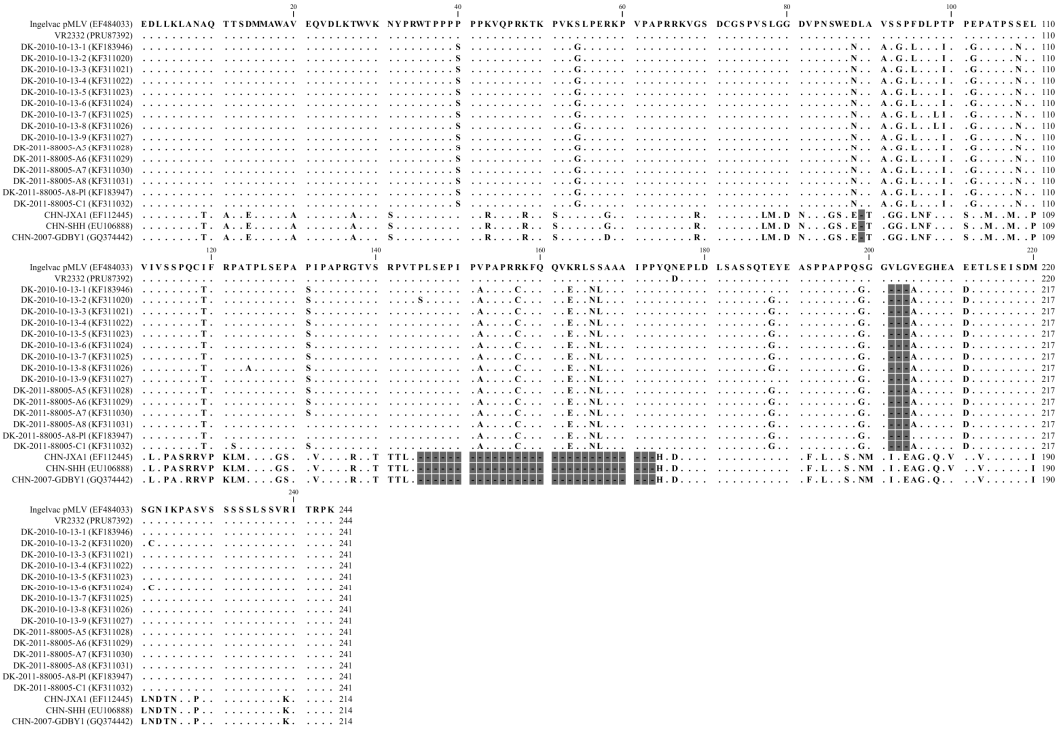


Fig. 3. Diagram of the mean body temperature measurements

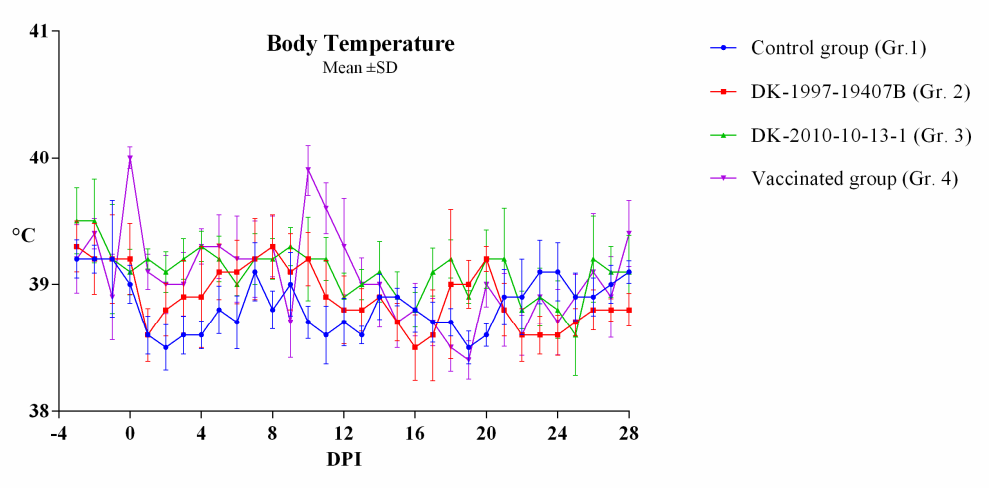


Fig. 4. Diagram of mean white blood cell (WBC) count

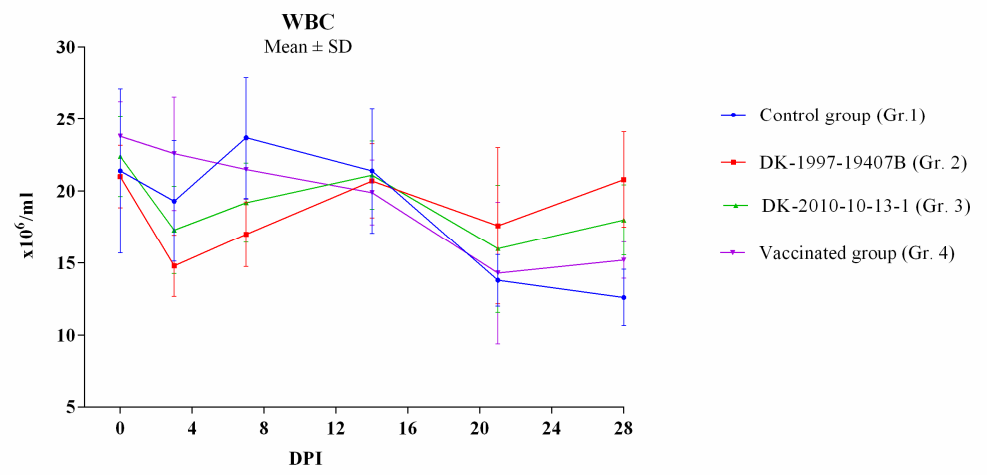


Fig. 5. Mean viral load in serum quantified by real-time RT-PCR

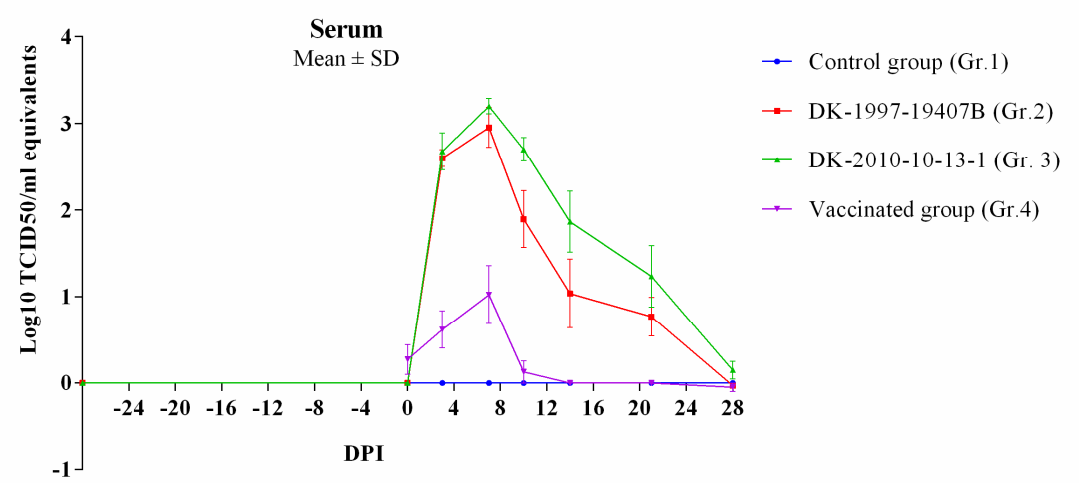


Fig. 6. Diagram of mean viral load quantified by real-time RT-PCR

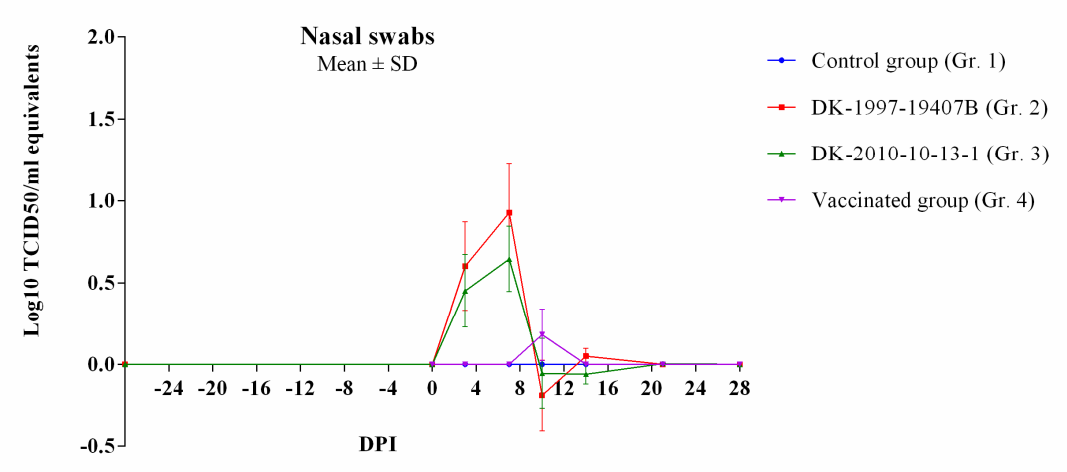


Fig. 7. Results of the PRRSV blocking ELISA. A sample was considered positive when the blocking percentage was below 44%

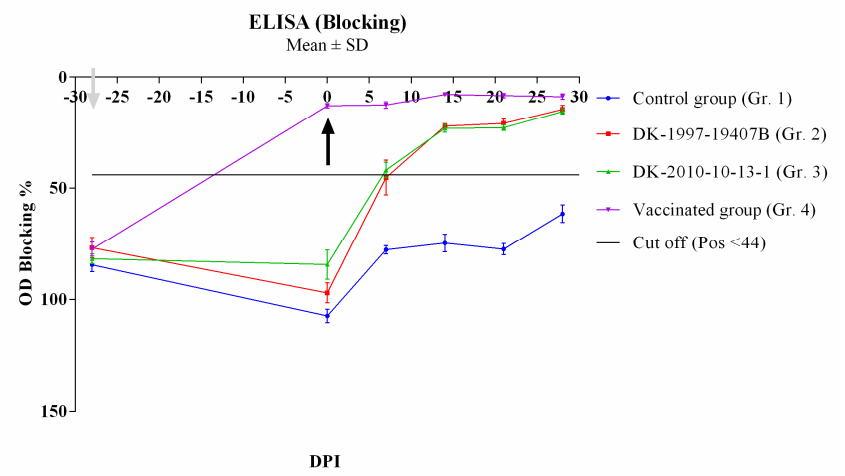


Fig. 8. Results of the IPMA analysis. The serum samples were tested in five dilutions (1:50-1:6250) and the results are expressed as the highest dilution given a positive signal

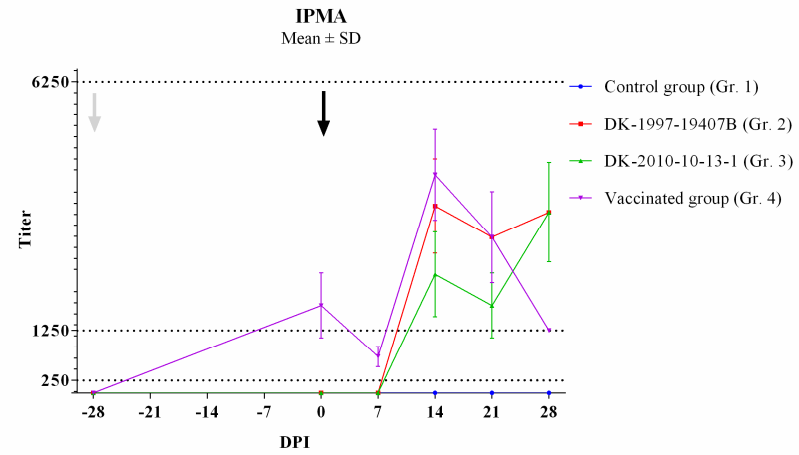


Fig. 9. Results of the CRP analysis (group means) expressed as ng/ML

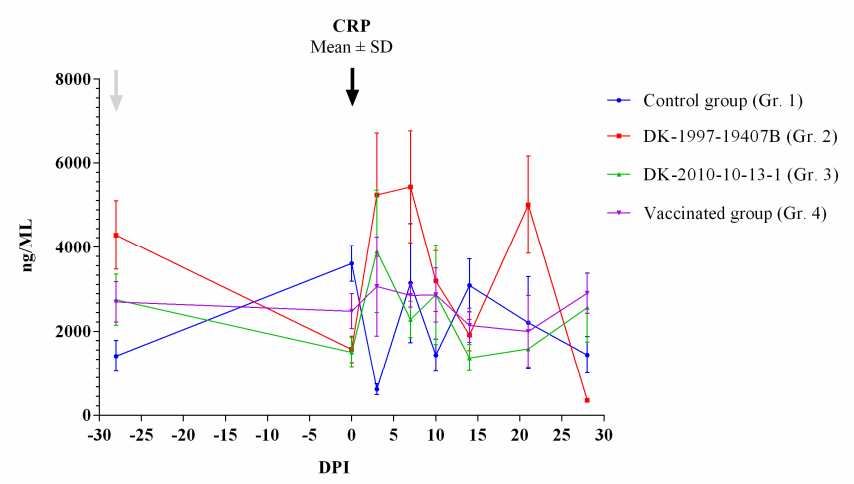
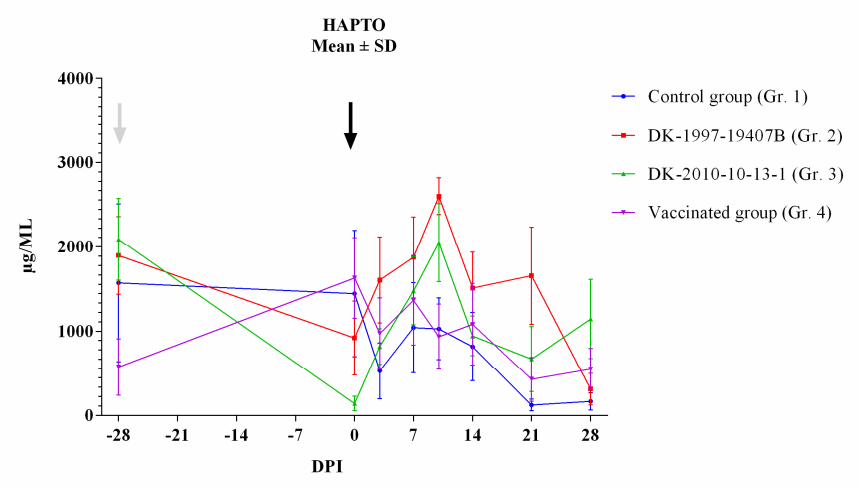


Fig. 10. Results (groups means) of the haptoglobin analysis expressed as µg/ML



DISCUSSION, CONCLUSION, AND PERSPECTIVES

The main focus of this PhD project has been on the genetic diversity of the Porcine Reproductive and Respiratory Syndrome Viruses circulating in the Danish pig population. To accomplish this, optimization of the amplification method of smaller genes for cycle sequencing was performed and a method for sequencing the complete PRRSV genome from long range PCR fragments using next generation sequencing technologies was developed. Furthermore, an experimental study of a Type 2 PRRS virus causing significant clinical disease in the field was carried out. The method for complete genome sequencing was used to sequence the case virus.

In this project, traditional cycle sequencing based on the Sanger method (Sanger et al., 1977) was used for the sequencing of shorter regions of the PRRSV genome. For this purpose cycle sequencing was an obvious choice as it is fast and obtainable for most laboratories. However, cycle sequencing has its limitations when it comes to sequencing length and sequencing depths. To sequence the complete genome of PRRSV using cycle sequencing the genome has to be covered by PCR fragments of length adequate for this sequencing method (~900 bp). Due to the high diversity between PRRS viruses it is difficult to design conserved primers matching all PRRSV genomes, hence longer PCR fragments are preferred. When using next generation sequencing technologies the lengths of the PCR fragments are of no concern and long amplicons are applicable. So it was with the use of NGS technologies for sequencing in mind the method for long range PCR amplification was developed. First, the four-fragment amplification strategy was developed and then the idea came of using some of the primers from the four-fragment amplification to cover the PRRSV genome in only two PCR fragments. However, the amplification of PRRSV in two fragments does not work on all samples and the output may be lower than when amplifying the genome in 4 fragments and therefore are the majority of the complete genomes obtained during this PhD project from sequencing on four PCR fragments. The two fragment strategy may still be a good choice for some applications (cloning of the virus, quasispecies experiments, test for recombination etc.) however individual optimization for each virus to be sequenced may be necessary to gain a higher concentration of the PCR amplicons.

The data output or depth of coverage obtained from next generation sequencing is the most pronounced improvement when comparing to cycle sequencing. From the three NGS platforms used during this project the average time a single nucleotide was read during the sequencing process were ~10,000 times for the Illumina HiSeq2000 and ~250 times for Roche 454 FLX and Ion

Torrent PGM™ Sequencer. The obtained sequencing depth can be increased for Roche and Ion Torrent by reducing the number of isolates pooled prior to sequencing although this will increase the cost per virus. The sequencing depth can be used to study genetic variation in relation to a reference sequence or the existence of quasispecies, however, precautions should be taken when determining this as the variations seen may have originated from errors induced during the RT and PCR steps and not during replication of the virus in the host. New quasispecies may also be introduced when propagating PRRSV *in vitro*. Protocols exist that generate complete genomes using NGS performed directly on RNA without an amplification step. In most cases this procedure requires, however, that the virus have been propagated. The method described here allows full length sequences to be generated directly from tissue or serum and do not require a large virus load to be present.

From the study of Type 1 PRRS viruses circulating in the Danish pigs, it was confirmed that only subtype 1 is present in Denmark. However, the diversity between the Type 1 PRRS viruses was relatively high and phylogenetic analysis of ORF5 and complete genomes showed that the Danish Type 1 PRRS viruses divided into two clusters, one LV/vaccine-like strain and one resembling an early introduced strain. The vaccine-like strain was the dominating type of Type 1 PRRSV following the introduction of Porcilis PRRS in the beginning of 2001.

The study of Type 2 PRRS viruses was a priority as Denmark was the first country in Europe to be infected with this genotype in relation to a vaccination program with a live attenuated vaccine (Botner et al., 1997, Madsen et al., 1998). Furthermore, it was interesting to study the evolution of the vaccine strain in a country with minimal import of living pigs. Only a scarce number of Type 2 PRRSV ORF5 sequences obtained from viruses isolated in Europe have been published (Stadejek et al., 2013) and no complete sequences have been sequenced and published before the present PhD project started. From the diversity study of Danish isolated Type 2 PRRS viruses it was found that they all were classified to a single cluster (sub-lineage 5.1 (Shi et al., 2010b)) which comprised strains closely-related to the Type 2 protogenotype isolate VR2332 and the modified live vaccine strain. However, the diversity between the Danish viruses was as high as 9.1 % indicating pronounced genetic drift of Danish Type 2 PRRS viruses.

From the results obtained from both diversity studies there were no indications of new introductions of PRRSV strains into the Danish pig population and on the basis of these finding the currently used diagnostic tools should be able to detect the PRRS viruses infecting the Danish pigs. However, an important point that has to be considered is that currently the only method used for

virus detection is real-time RT-PCR. If a diverse PRRSV stain is introduced, the standard primer and probes may not detect the new virus. Before implementing real-time RT-PCR as a diagnostic tool for detection of PRRSV, virus detection was carried out by inoculation on cell cultures followed by immunohistochemical staining. However this method has disadvantages as well, besides being slow and time-consuming, not all PRRS viruses are able to proliferate *in vitro* either due to incorrect handling of the sample material from the herd to the laboratory or because the virus is not adaptable to cell cultures. To overcome both scenarios, both methods could be implemented in the diagnosis of PRRSV. Another solution could be to develop a multiplex PCR assay where several primer pairs and probes that were able to cover a broader range of variant templates where used. To design primer and probes that fulfills this, it is very important to be aware of which viruses circulating in other countries as well and also participation in ring tails are indispensable. This further signifies the importance of continuing surveillance of the diversity of PRRSV also after this PhD study and the end of the EU project in 2014. Hopefully the industry is willing to support this activity.

PRRS is a notifiable disease in Denmark in case the virus has been detected by real-time RT-PCR, culturing or detection of antibodies against PRRSV in serum or pleura obtained from stillborn fetuses or piglets before ingestion of colostrum. As a consequence, the herd cannot deliver pigs to normal slaughterhouses for six months which leads to significant economical losses. These regulations do not apply for detection of antibodies in adult pigs. These regulations causes the field veterinarians and pig producers to hesitate submitting samples for virus detection, hence the numbers of acute infected herds in Denmark may be higher than what is believed on the background of positive cases determined from virus detection by real-time RT-PCR. Since most Danish herds use serology even for diagnosis of acute cases of PRRSV further characterization of the virus isolates by sequencing are not possible. Thus, a new variant of PRRSV could easily escape detection in Denmark, emphasizing that some degree of active surveillance is needed.

During the course of the PhD project a natural infection with Type 2 PRRSV occurred in the Northern part of Denmark. This herd experienced significant clinical signs including reproductive failures in sows and high mortality rates for weak born piglet. On the basis of the clinical signs, it was feared that a new and more virulent strain of PRRSV was introduced into Denmark. In connection to this infection, the newly developed methods showed its importance and practical applicability. Sequence analysis on samples from this herd revealed that the strain was similar to other Danish viruses but also that it had some special genetic features in some minor genes.

Subsequently an experimental infection with this strain revealed that the infection dynamic was similar to circulating classical Danish Type 2 strains. This case underlined perfectly that methods for fast and reliable sequencing of PRRSV isolates are important tools for the elucidation of unusual events that in turn could treating the important export of living pigs and pork product to PRRSV "sensitive" countries such as Brazil and China.

In conclusion, the results obtained during this project showed homology between viruses within the same genotype. On this background there is no reason to question the ability of the currently used diagnostic tools to detect PRRSV and the available vaccines should also be protective against present day Danish PRRS viruses. However, the genetic heterogeneity between the two genotypes, Type 1 and Type 2, are so distinct that perhaps PRRSV Type 1 and PRRSV Type 2 should be regarded as two different viruses. This heterogeneity is also very pronounced in an antigenic manner, as amino acid compositions at neutralizing epitopes greatly differs. Recombination between PRRS viruses have been documented on several occasions (Chen et al., 2013, Forsberg et al., 2002, Frossard et al., 2013). However, proof of recombination between Type 1 and Type 2 PRRSV strains has of this date never been published and since recombination preferentially occurs between genomes with high similarity it further demonstrate the differences between the PRRSV genotype 1 and 2.

A future study regarding PRRSV could be to pursue the thought of dividing Type 1 and Type 2 into two distinct PRRS viruses. Recombination studies should be performed *in vivo* and *in vitro*, using co-infection with both genotypes followed by analysis of recombination between the two viruses using the described methods for complete genome sequencing. A resembling *in vitro* study has been carried, however only a small region was sequenced and no indication of recombination between Type 1 and Type 2 was detected (van Vugt et al., 2001).

A further optimization of complete genome sequencing directly from RNA preferable extracted from primary infected material offer a new and unique tool to study the genetic variation and quasispecies even without cDNA steps.

One of the milestones in understanding PRRS would be to identify virulence marker(s). From the complete genome analysis and experimental infection with a case virus given severe clinical disease in the field, failed to identify a virulent marker. This further underlines the difficulties in predicting the outcome of PRRS and more than one field incident of significant clinical disease are needed to be able to compare viruses. To investigate potential virulent markers it is of great importance that the clinical signs of the virus in the field are known together with information on co-infections in

the herd. Luckily, the National Veterinary Institute already have a splendid collaboration with the Danish field veterinarians and information of the clinical signs incurred from recently infected herds should be obtainable.

Reverse genetics could also be a very interesting field to further investigate the virulence of PRRSV by testing specific motifs identified in hot field strains.

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APPENDIX I

Introduction to own work

In the following sections thorough descriptions of the laboratory methods used during the PhD project are presented. All the described methods, together leads to the main objective of the study: to investigate the genetic diversity of PRRSV circulating in Danish pigs.

Methods

Marc-145 cells

One ampule of frozen Marc-145 cells (1.6 x10^6 cells/ml) was thawed on a 37°C water bath. Immediately after, the thawed cell suspension was transferred to a 15 ml Nunc tube added 10 ml wash medium (app. I. table 1). The cell suspensions were centrifuged at 700 x g for 15 minutes. The supernatant was discarded and the cell pellet was resuspended in 40 ml initial growth medium and transferred to 160 ml cell culture flask. The cells incubated in a cell incubator at 37°C and 5 % CO_2. It took 1-2 days for the cells adhere. The 10 % FCS maintenance medium (app. I. table 1) was changed every second day.

When confluent the cells were trypsinized. First the cell culture medium was discarded and the cells were rinsed 3 x 3 ml 100µg/ml Trypsin. Following the third rinse, 1 ml of Trypsin was left in the cell culture flask and the cells incubated at 37°C until they had loosened. The cells were added 5 ml of maintenance medium and 1 ml cell suspension (1:6 split) was transferred to a new cell culture flask containing 40 ml of 10 % FCS maintenance medium. The cells were incubated in a cell incubator at 37°C and 5 % CO_2. The cells adhered within 24 hours. Cells of a 1:6 split usually takes 5 days to grow to confluence.

Freezing Marc-145 cells for back-up/storage was done by transferring trypsinized cells to a 50 ml tube added 40 ml wash medium. The cell suspension was mixed by roughly pipeting up and down. The cells in suspension were counted using a hemocytometer and the rest of the cell suspension was centrifuged at 700 x g for 15 minutes. The cell pellet was resuspended in freezing medium (app. I. table 1) to a final cell concentration of approximately 1.5 x 10^6 cells/ml. The cell suspension was divided in 1 ml aliquots to cryotubes. The cell aliquots were stored at -80°C for 24 hours following transfer to N_2-tank for storage.

App. I. table 1. Cell culture media for continuous cell lines

MEM cell medium	FCS final conc. (%)	L-glutamine final conc. (mM)	NEAA final conc. (%)	Pen/strep final conc. (mg/ml)	DMSO final conc. (%)
Wash medium	2	-	-	-	-
Initial growth medium	20	2	1	0.1	-
Maintenance medium	5/10	2	1	0.1	-
Freezing medium	20	-	-	-	10

Porcine alveolar macrophages

The PAM ampules were transported on dry ice from the N_2-tank to the cell laboratory. The frozen PAM ampules were quickly thawed on a pre-heated 37°C water bath. Immediately following thawing the PAMs were divided between two 50 ml tubes containing 30 ml wash medium (app. I. table 1) and centrifuged at 1000 x g for 10 minutes, 4°C. Following centrifugation the supernatant was discarded and the cell pellet was resuspended in 5 % FCS maintenance medium (app. I. table 1) to the cell concentration needed. For propagation of PRRSV, the cell suspension was prepared as a 2.0 x 10^6 cells/ml. After adding the PAM suspension to e.g. 24-well cell culture plates, the cells were incubated in a cell incubator at 37°C and 5 % CO_2 for 1 hour where after the cells had settle and ready for inoculation with PRRSV.

Propagation of PRRSV on PAM and Marc-145 cells

For inoculation on PAMs, cells were seeded at the concentration of 2.0 x 10^6 PAMs/ml. For inoculation on Marc-145 cells, the cells had to be confluent or close to.

Inoculation material:

Pleura: dilute 1:5 in MEM supplemented with 10 x pen/strep.

Lung: homogenize tissue in 1:10 MEM supplemented with 10 x pen/strep.

Serum: use as it is, although serum is not the best inoculation material for propagation of PRRSV.

All inoculation materials were sterile filtrated through a 0.45 µM sterile filter.

For inoculation in 24-well plates lung and pleura was added in 200µl/well and serum was added in 50µl/well.

Following inoculation with the sample material, cells incubated in a cell incubator at 37°C and 5 % CO_2. CPE could be seen 3-5 day after inoculation. When CPE was observed the propagation of virus was terminated by transferring the cell medium supernatant to storage tubes and stored at -

80°C. A second passage could be carried out by transferring 50 µl of cell medium supernatant to new cells.

To confirm propagation of PRRSV, RNA was extracted from the cell medium supernatant and screened by PriProET RT-PCR.

Extraction of RNA

Total RNA was extracted from lung tissue, pleura, nasal swabs, serum, and cell culture supernatant using commercial RNA purification kits from QIAGEN.

Lung tissue was homogenized as follows:

1. RLT buffer (included in RNeasy Minikit, QIAGEN) was added β-mercaptoethanol to a 1 % concentration.

2. 2 ml tubes were prepared with one 5 mm steal beads per tube and 1400 µl RLT- β-mercaptoethanol buffer.

3. 70 mg lung tissue was cut into small bits and added the 2 ml tubes

4. The lung tissue was homogenized using Tissuelyser II (QIAGEN) for 3 minutes at 30 Hz. If needed, the procedure was repeated.

5. The tubes were centrifuged at 12,000 x g for 3 minutes.

6. Carefully the supernatant was pipette in two aliquots of 600 µl to new 1.5 ml eppendorf tubes. One tube was saved as back-up at -20°C.

Total RNA extraction from lung homogenate, nasal swabs, and pleura using RNeasy Minikit (QIAGEN):

Positive controls were prepared from 200 µl PRRSV cell culture supernatant added 400 µl RLT-β-mercaptoethanol buffer.

Negative controls were prepared from 200 µl RNAse free water added 400 µl RLT- β-mercaptoethanol buffer.

Nasal swabs and pleura samples were prepared from 200 µl material added 400 µl RLT- β-mercaptoethanol buffer.

1. Each sample (600 µl) was added 1 vol. 70 % EtOH and mixed by pipeting.

2. 700 µl sample was added the RNeasy mini column and centrifuged at 8,000 x g for 15 seconds. The flow-through was discarded and the rest of the sample material was added the column and the centrifugation was repeated.

3. 700 ml RW1 buffer was added to the column. The samples were centrifuged at 8,000 x g for 15 seconds. The flow-through was discarded.

4. 500 µl RPE buffer was added the column and centrifuged at 8,000 x g for 15 seconds. The flow-through was discarded.

5. An additional 500 µl RPE buffer was added the column and centrifuged at 8,000 x g for 2 minutes. The flow-through was discarded.

6. Centrifuged the column at 12,000 x g for 1 minute followed by transfer of the column to a new 1.5 ml eppendorf tube.

7. 60 µl RNAse free water was carefully added to the center of the column and incubated for 1 minute at RT. The RNA was eluted by centrifugation at 8,000 x g for 1 minute.

8. The purified RNA was stored at -80°C or evaluated by PriProET RT-PCR.

Total RNA extraction from serum and cell culture supernatant using QIAamp Viral Minikit (QIAGEN):

1. 140 µl serum or cell culture supernatant were added to 1.5 ml eppendorf tube.

2. 310 µg carrier RNA was dissolved in 310 µl AVE buffer (1 µg/µl).

3. The volume of AVL buffer needed for the RNA extraction and how much carrier RNA to add was calculated using the following equations:

$$n \times 0.56 \text{ ml} = y \text{ ml}$$

$$y \text{ ml} \times 10 \text{ µg/ml} = z \text{ µl}$$

n = number of samples to purify + 2

Y = total volume of AVL buffer

z = volume of 1 µg/µl carrier RNA to add to the AVL buffer

4. 560 µl AVL-carrier RNA buffer was added to the tubes containing the sample material and incubated for 10 minutes at RT.

5. The samples were centrifuged shortly.

6. 560 ml EtOH was added the samples and mix by pulse-vortex for 15 seconds.

7. 630 µl sample material was transferred to the QIAamp Viral RNA mini column and centrifuged at 6,000 x g for 1 minute. The flow-through was discarded.

8. The rest of the sample material was added to the column and the centrifugation step was repeated.

9. 500 µl AW1 buffer was added to the column and centrifuged at 6,000 x g for 1 minute. The flow-through was discarded.

10. 500 ml AW2 buffer was added to the column and centrifuged at full speed for 3 minutes. The flow-through was discarded.

11. The column was centrifuged at full speed for 1 minute following transfer of the column to a new 1.5 ml eppendorf tube.

12. 60 µl RNAse free water was carefully added to the center of the column and incubated at RT for 1 minute. The RNA was eluted by centrifugation at 6,000 x g for 1 minute.

13. The purified RNA was stored at -80°C or evaluated by PriProET RT-PCR.

Primer Probe Energy Transfer (PriProET) RT-PCR

The extracted RNA was screened for PRRSV by a modified PriProET real-time RT-PCR assay (Balka et al., 2009). The real-time RT-PCR kit used was the Ultrasense onestep quantitative RT-PCR system from Invitrogen. The real-time RT-PCR was carried out in a total reaction volume of 25 µl containing 2 µl extracted RNA. The primer and probes used are listed in app. I. table 2, the mixture proportions of the PCR reaction mix is listed in app. I. table 3, and the RotorGeneQ real-time PCR machine thermal cycle program is listed in app. I. table 4.

App. I. table 2. Primer and probes used for screening RNA for PRRSV

Name	Orientation	Sequence 5'-3'
Primer 1	Forward	AGCCTCGTGYTGGGYGGCARA
Primer 2	Reverse	(FAM)-TCAGCAWYTGRCACAGYTGAT
Probe Type 1 PRRSV	Forward	TCCGATGGGGAATGGCCAGCCAGTCT-(ATTO663)
Probe Type 2 PRRSV	Forward	AAAGAAGGGGGATGGCCAGCCAGTCT-(ATTO663)

Primer 1, Primer 2, and Probe Type 1 PRRSV sequences are from Balka et. al. (2009). Probe Type 2 PRRSV was designed by colleges in the virology group.

App. I. table 3. Ultrasense onestep quantitative RT-PCR system reaction mix

Reagent	Final concentration	Volume (µl)
RNAse free water		13.94
RNA Ultrasense 5x reaction mix	1x	5.0
Primer 1 (10 µM)	0.4 µM	0.31
Primer 2 (10 µM)	1.0 µM	1.25
Probe Type 1 or Type 2 (ATTO663) (10 µM)	1.0 µM	1.25
RNA Ultrasense Enzyme Mix	1x	1.25
Final volume		**23.0**

App. I. table 4. The RotorGeneQ real-time PCR machine thermal cycle program

PCR step	Temperature °C	Duration
Calibration	55	
RT-PCR	50	30 minutes
Denaturation	95	2 minutes
50 cycles:		
Melt	95	15 seconds
Annealing	55	30 seconds
Elongation	72	20 seconds
Hold	72	5 minutes
Hold	95	60 seconds
Melt	50°C -> 90°C w. 1°C rise per step	

The Ct values were determined and values below Ct: 38 was determined to be positive for PRRSV. From the melting curve and comparisons with the positive controls the PRRSV genotype could be deduced.

cDNA synthesis

Complete genome cDNA was synthesized from PRRSV RNA samples using SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen (Rasmussen et al., 2008, Rasmussen et al., 2010).

Two mixtures were prepared as listed in app. I. table 5. Eight (8) µl RNA incubated with mixture 1 at 65°C for 5 minutes. Then the PCR tube was placed on ice for 1 minute. Mixture 2 was added mixture 1 and the cDNA synthesis was carried out at 50 °C for 90 minutes followed by 85°C for 5 minutes. The PCR tube was briefly centrifuged and added 1 µl RNase H. The RNase reaction was carried out at 37°C for 20 minutes. The cDNA was used for conventional PCR and stored at -20°C.

App. I. table 5. cDNA synthesis mixtures

Reagents	Volume (µl)
Mixture 1:	
10 µM RT-primer (Nielsen et al., 2001)	1
10 mM dNTPs	1
Final volume	**2 µl**
Mixture 2:	
10x RT buffer	2
25 mM $MgCl_2$	4
0.1 M DTT	2
RNAseOUT	1
Superscript III RT	1
Final volume	**10 µl**

Conventional PCR

Conventional PCR was carried out using the AccuPrime Taq DNA Polymerase High Fidelity Kit from Invitrogen and complete genome cDNA as template. Short and long range PCR amplicons were produced for cycle sequencing and next generation sequencing, respectively. Primers for the shorter PCR amplicons, ORF2-7 for Type 1 PRRSV and ORF5, ORF7, and partial NSP2 for Type 2 PRRSV are listed in app. I. table 6. For the primer sequences for the long range PCR amplicons please see table 2 in Manuscript I.

App. I. table 6. Primers used for ORF2-7, ORF5, ORF7, and partial NSP2 amplification

Primer name	Forward primer (5'-3')	Reverse primer (5'-3')
Type 1 PRRSV:		
ORF2-EU	CTG GCA CAG AAT TGC AGG TA	GCA CAC TGA TGA GCC ATT GT
ORF2-3-EU	TGC TCC GCG CTT CTC CGT TCG	ACA TAG CGT AGA GCT GGA ATT CG
ORF3-EU	ACA ATG GCT CAT CAG TGT GC	TGA AGC CTT TCT CGC TCA TT
ORF4-EU	AGC GTG ACC ATG ATG AGT TG	AAA AGC CAC CAG AAG CAA GA
ORF5b-EU	TGA GGT GGG CTA CAA CCA TT	AGG CTA GCA CGA GCT TTT GT
ORF6-EU	GTC CTC GAA GGG GTT AAA GC	CTG TCC TCC CCT AGG TTG CT
ORF7b-EU	GGC AAA CGA GCT GTT AAA CG	AAT TTC GGT CAC ATG GTT CC
Type 2 PRRSV:		
ORF5-US	GCT CCA TTT CAT GAC ACC TG	AAA GGT GCA GAA GCC CTA GC
ORF7-US	GCC CCT GCC CAI CAC G	TCG CCC TAA TTG AAT AGG TGA
NSP2-partial	CTT AAA GAC CAG ATG GAG GAG G	CGA TGA TGG CTT GAG CTG AGT AT

Primer sequences for Type 1 PRRSV were from Diaz et al. (2006). Primer sequences for ORF5-US and ORF7-US were from Oleksiewicz et. al. (1998). Primer sequences for NSP2-partial were from Zhou et al. 2009.

The conventional PCR reaction was carried out in a final reaction volume of 50 µl containing 2 µl cDNA template. The mixture proportions for the AccuPrime Taq DNA Polymerase High Fidelity Kit are listed in app. I. table 7.

App. I. table 7. AccuPrime Taq DNA Polymerase High Fidelity reaction mix

Reagent	Volume (µl)
RNAse free water	40.5
10x AccuPrime PCR buffer I	5
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
AccuPrime Taq High Fidelity	0.5*
cDNA	2
	50 µl

*For PCR amplicons covering the PRRSV genome in two fragments 1 µl polymerase is used.

The conventional PCR was carried out on a T3 Thermo cycler (Biometra) with the PCR cycling program as listed in app. I. table 8. The PCR products were analyzed by agarose gel electrophoresis using E-gel® 0.8 % or 2 % agarose gels from Invitrogen. Five µl of PCR product mixed with 7 µl 1:40 TrackIt™ loading buffer (Invitrogen) were loaded onto the gel. 10 µl 1 kb Plus DNA ladder (Invitrogen) or 100 bp DNA ladder (New England BioLabs) was used as size marker.

App. I. table 8. PCR cycling program for conventional PCR amplification

PCR step	Temperature (°C)	Duration
Denaturation	94	15 seconds
45 cycles of:		
Melt	94	15 seconds
Annealing	55*	30 seconds
Elongation	68	1 minute per Kb elongation
Hold	68	2 minutes per Kb elongation (min. 5 minutes)
Hold	4	∞

*The annealing temperature can be lowered to e.g. 52°C when amplifying long range amplicons covering the PRRSV genome in two fragments.

Purification of PCR amplicons

Prior to cycle or next generation sequencing, primers and buffers from the PCR reaction needed to be removed which was done by purifying the PCR products using Roche's High Pure PCR Product Purification kit as follows:

1. The PCR reaction volume was adjusted to 100 µl with Milli Q water (MQ) in a 1.5 ml eppendorf tube.
2. 500 µl Binding buffer was added the tube with the PCR reaction.
3. The solution was mix by vortexing followed by a quick spin.
4. The sample was transferred to a High Pure filter tube placed in a collection tube.

5. The sample was centrifuged at full speed for 1 minute and flow-through was discarded.

6. 500 µl Wash buffer was added the filter tube and centrifuged at full speed for 1 minute. The flow-through was discarded.

7. An additional 200 µl Wash buffer was added, the filter tube was centrifuged as before and the flow-through was discarded.

8. The filter tube was transferred to a 1.5 ml eppendorf tube and carefully added 30 µl 50°C pre-heated RNAse free water to the center of the filter and then incubate for 1 minute at RT.

9. The PCR amplicon was eluted by centrifugation at full speed for 1 minute.

10. The concentration of the purified PCR amplicon was determined using a spectrophotometer.

For removal of unspecific PCR amplicons, PCR amplicon of the right molecular size was extracted from the gel.

1. The PCR amplicon was cut from the gel and placed in a 1.5 ml eppendorf tube.

2. 3 µl Binding buffer from the High Pure PCR Product Purification Kit (Roche) was added for each mg of gel slice.

3. The mixture was vortexed and incubated at 56°C for 10 minutes with a brief vortex every 2-3 minutes.

4. Following dissolving of the gel slice the sample was added 1.5 µl isopropanol for every mg of gel slice. The sample was vortexed thoroughly.

5. The rest of the purification followed from section 4 in the first purification protocol described.

Cycle sequencing

Cycle sequencing was used for the sequencing of shorter PCR amplicons, e.g. ORF2-7 and partial NSP2.

The sequencing primers were the same as the primers used for the PCR amplification (app. I. table 6).

200 ng of purified PCR amplicon in 10 µl MQ was prepared with 4 µl 5 µM sequencing primer and send to LGC Genomics GmbH (Berlin, Germany) for sequencing. The obtained raw data was assembled into contigs using the commercial software CLC Main workbench v. 6.6.2 (CLC BIO, Aarhus, Denmark).

Next generation sequencing

Complete genome sequences of PRRSV were sequenced from long range PCR amplicons covering the full genome in two or four fragments using next generation sequencing (NGS) technologies.

For the sequencing of PCR amplicons covering the complete genome of PRRSV in two fragments, 2.7 µg in total of equimolar concentration were sent to LGC Genomics GmbH (Berlin, Germany) for sequencing on the platform Roche 454 FLX.

The sequencing of PCR amplicons covering the PRRSV genome in four fragments, two NGS technologies were utilized; Illumina HiSeq2000 (ARK genomics The Roslin Institute, University of Edinburgh) and Ion Torrent GPM sequencer (DTU Multi-Assay Core (DMAC), Kgs. Lyngby, Denmark).

For sequencing on the Illumina® HiSeq 2000 platform, libraries were prepared from the Illumina® TruSeq™ DNA Sample Preparation v2 Kit A and B (Illumina®, cat. no. FC-121-2001-1 and FC-121-2002) following the TruSeq DNA Sample Preparation v2 Guide with few modifications.

Fragmentation of PCR amplicons:

All 4 PCR amplicons covering the whole genome of one PRRS virus were pooled in equimolar quantity to a final amount of 1.1 µg and added 10 mM Tris-Cl, pH 8.5 to a final volume of 55 µl. The pooled PCR fragments were sheared into fragments with an average size of 250 bp in a Covaris™ microTUBE with AFA fiber (Covaris, cat. no. 520045) on a Covaris™ S2 Ultra-sonicator using the following settings: Duty cycle: 10 %, Intensity: 5.0, Cycles/Burst: 200, and Duration: 105 seconds.

End repair:

The dsDNA 5’ or 3’ overhangs generated from Covaris shearing was repaired with NEBNext® End Repair Module (New England BioLabs®inc., cat. no. E60510S). 50 µl of sheared PCR amplicons were mixed with 1 µl E. coli DNA Ligase for Fragmentase (New England BioLabs®inc., cat. no. M0348L), 10 µl NEBNext End Repair Reaction Buffer (10x), 5 µl NEBNext End Repair Enzyme Mix, and MQ water to a final volume of 100 µl. Incubate in a thermal cycler for 30 minutes at 20°C following column purification using MinElute PCR Purification Kit (Qiagen, cat. no. 28004) eluting twice in 10 µl and 7.5 µl elution buffer.

Adenylate 3' ends:

To adenylate 3' ends, 15 µl of purified end repaired sample was mixed with 12.5 µl A-Tailing Mix, and 2.5 µl Resuspension Buffer incubating at 37°C for 30 minutes.

Ligate adaptors:

To each sample 2.5 µl Resuspension buffer, 2.5 µl Ligation Mix, and 2.5 µl of specific DNA Adaptor index were added and mixed by gentle pipeting the entire volume up and down 10 times. The samples were incubated on a pre-heated thermal cycler at 30°C for 10 minutes. Following incubation the samples were added 5 ml Stop Ligation Buffer and mixed thoroughly by pipeting up and down 10 times. The samples were using MinElute PCR Purification Kit eluting twice in 10 and 12.5 µl elution buffer.

Size selection:

For size selection the gel method was used. A 2 % Agarose-HR gel with GelRed was made as follows: 3 g Agarose-HR was added 150 ml 1x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, pH 8.0) and dissolved by heating in a microwave oven. Cool down a few minutes then add 15 µl 1x10.000 GelRed Nucleic Acid Stain (Biotium, cat. no. 41003) and swirl to mix. Load 20 µl purified adaptor ligated sample with 7 µl loading buffer (50 mM Tris, pH: 8.0, 40 mM EDTA, 40 % w/v sucrose) and 5 µl diluted (8 µl ladder + 3 µl loading buffer) TrackIt™ 1 kb plus ladder (Invitrogen, cat. no. 10488-085) and run the gel for 2 hours at 120 V constant voltage.

Excise the band from the gel that corresponds to the size determined following fragmentation added the 100 bp for the ligated adaptors. Purify the gel slices using QIAquick Gel Extraction Kit (QIAGEN, cat. no. 28704) and elute in 25 µl elution buffer.

Enrich DNA fragments:

Proceeding to the enrichment of the DNA fragments by PCR. The samples were added 5 µl PCR Primer Cocktail and 25 µl PCR Master Mix and mixed well. The PCR reaction was carried out as follows: [98°C for 30 seconds], 12-16 cycles: [98°C for 10 seconds, 60°C for 30 seconds, 72 °C for 30 seconds] then finalize with 72°C for 5 minutes and cool down to 10°C. Following enrich PCR, the library samples were purified using Agencourt AMPure XP beads (Beckman Coulter, cat. no A63881). Transfer the library samples to a 96-well plate. Vortex the Agencourt AMPure XP bead

until well dispersed, then add beads 1:1 to library samples and mix well by gently pipeting. Incubate at room temperature for 15 minutes then place the plate on a magnetic stand for 2 minutes at RT or until the liquid appears clear. Remove and discard the supernatant with the 96-well plate still on the magnetic stand and wash the beads twice with 80 % freshly prepared EtOH. Remove the 96-well plate from the magnetic stand and let the plate stand at RT for 15 minutes to dry. Resuspend the dried pellet in 32.5 µl 10 mM Tris-Cl, pH 8.5 buffer and incubate at RT for 2 minutes. Place the plate on the magnetic stand for 2 minutes or until the liquid appears clear. Transfer 30 µl of the purified enrich PCR products to a new tube.

Validation of libraries:

To verify the size and determined the approximate concentration of the library products an Agilent DNA 1000 chip (Agilent Technologies, cat. no. 5067-1505) was run on an Agilent Technologies 2100 Bioanalyzer using the Agilent DNA 1000 Kit (Agilent Technologies, cat. no. 5067-1504) following the Agilent DNA 1000 Kit Quick Start Guide. All samples with concentration above 10 nM were diluted to 10 nM.

qPCR:

To accurately quantify the number of amplified molecules in the library sample a qPCR was performed using KAPA Library Quantification Kit for Illumina Genome Analyzer platform (KAPA Biosystems, cat. no. KK4824). The library samples were diluted 1:3000 in Library Dilution Buffer (10 mM Tris-HCl, pH: 8.0 + 0.05 % Tween 20) in a deep well 96-well plate. The plate was sealed and incubated at RT on rocking table for 5 minutes. Followed the KAPA Library Quantification Kit detailed protocol for the qPCR set-up. The concentrations were calculated from equations' described in the KAPA Library Quantification Kit detailed protocol and further dilutions were carried out by the ARK genomic staff. The libraries were ready for cluster generation and sequencing on Illumina HiSeq 2000 by ARK genomics, Roslin Institute, University of Edinburgh.

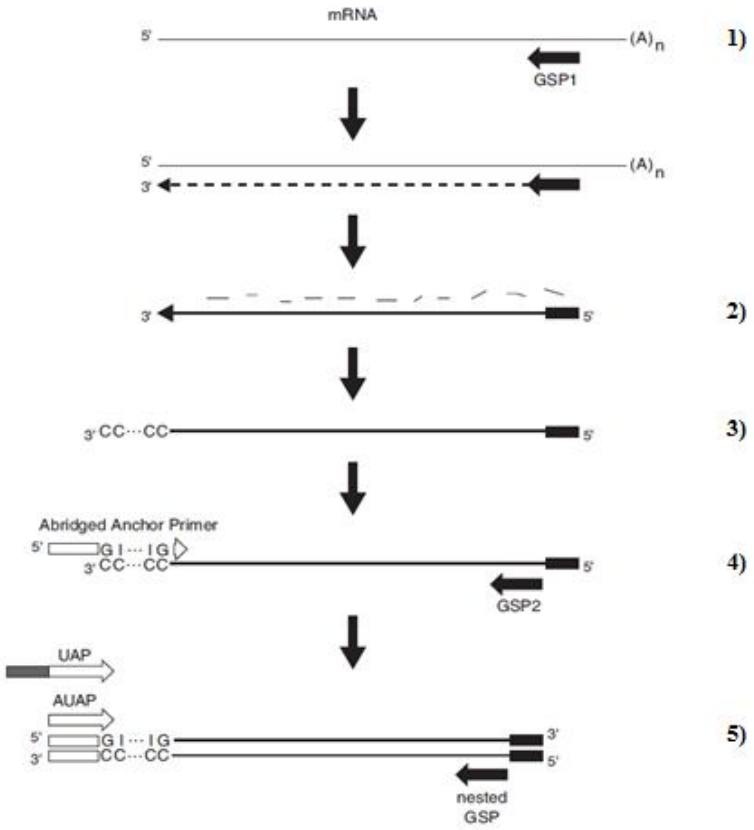
Sequence data analysis:

After initial removal of adaptors and low quality sequences, the quality of the FastQC files was examined using the applicant FastQC (version 0.10.1). The reads were trimmed in regards to the FastQC report. Trimming was done by the Prinseq-lite tool and mapping of the reads was

performed by the Burrows-Wheeler aligner (BWA) using the Aln algorithm. For further details on the data analysis and results please see Manuscript I.

5' RACE

For some Type 2 viruses, it was difficult to amplify the further most 5' end of the genome using the primes designed for long range PCR amplification in that area of the genome (see Manuscript I). Hence a method for rapid amplification of cDNA ends (RACE) was performed using Invitrogens 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (cat. no. 18374). An overview of the 5' RACE procedure is shown in app. I. fig. 1.



App. I. fig. 1. Overview of the 5' RACE procedure. *1) cDNA is synthesized from template RNA using a gene-specific primer as RT-primer (GSP1). 2) Degradation of RNA with RNAse mix. 3) 3' dC-tailing of purified cDNA. 4) PCR amplification of dC-tailed cDNA using the Abridge Anchor Primer and a second gene-specific primer (GSP2). 5) Re-amplifying PCR product obtained from step 4 using the AUAP primer and a third gene specific primer (nested GSP). The figure is from the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 protocol.*

The gene-specific primers for the 5' RACE were designed using the Primer3Plus web utility (Untergasser et al., 2007). The primer sequences are listed in app. I. table 9.

App I. table 9. Gene-specific primers for 5' RACE.

Primer name	Primer sequences (5'-3')
RT748 (GSP1)	CAA TGT CAT AGA CAG TAG
GSP2-538	TCA AGA CTG CAG GAG TGA GC
Nested-458	CCA CTG GTC ATT CGT GCG AT

Preparation before start:

Prepare 1X wash buffer:

1. Pipette 1 ml of the wash buffer concentrate into a 50 ml graduated cylinder
2. Add 18 ml of MQ and 21 ml of absolute ethanol (99 %). Mix well
3. Transfer to a blue cap bottle and store at 4°C

First strand cDNA Synthesis:

1. Prepare cDNA reagents A and B in two separate PCR tubes and keep reagent mix B on ice until use (app. I. table 10).

App. I. table 10. Mixture A and Mixture B for cDNA synthesis

Components (A)	Start concentration	Volume (µl)
RT748 (GSP1)	0.5µM	5.5
Template (mRNA)	~80 ng/µl	10
DEPH-treated water	-	-
Final volume		**15,5 µl**
Components (B)	**Start concentration**	**Volume (µl)**
PCR buffer	10X	2.5
$MgCl_2$	25 mM	2.5
dNTP mix	10 mM	1
DTT	0.1 M	2.5
SuperScript II	-	1
Final volume		**9.5 µl**

2. Incubate mixture A for 10 minutes at 70°C and place on ice when finished.
3. Collect mixture A by briefly centrifugation and add mixture B to mixture A.
4. Incubate the mixture in a thermo cycler at 42°C for 50 minutes followed by 70°C for 15 minutes (step 1, app. I. Fig. 1).
5. Add 1 µl RNAse mix, mix gently but thoroughly, and incubate at 37°C for 30 minutes. Collect the reaction by briefly centrifugation and place on ice (step 2, app. I. Fig. 1).

6. The procedure can be stopped at this point by storing the cDNA at -20°C or proceed to the next step.

S.N.A.P Column purification of cDNA:

1. Equilibrate the binding buffer to room temperature and cool the wash buffer to 4°C before starting the purification of cDNA.

2. For each sample to be purified, equilibrate ~100 µl RNAse free water at 65°C for use in step 8.

3. Add 120 µl binding solution (6 M NaI) t the cDNA reaction.

4. Transfer the cDNA/NaI solution to a S.N.A.P. column. Centrifuge at 13,000 x g for 20 seconds. Discard the flow-through.

5. Add 400 ml of cold 1X wash buffer to the spin column. Centrifuge at 13,000 x g for 20 seconds. Discard the flow-through. Repeat this step an additional three times.

6. Wash the column with 400 µl cold 70 % EtOH. Centrifuge at 13,000 x g for 20 seconds and discard the flow-through. Repeat the step once more.

7. Centrifugate the column at 13,000 x g for 1 minute and discard the flow-through.

8. Transfer the column to a 1.5 ml eppendorf tube and add 50 µl 65°C pre-heated RNAse free water to the column and centrifuge at 13,000 x g for 20 seconds.

9. The procedure can be stopped here by storing the purified cDNA at -20°C or proceed to the next step.

dC tailing of cDNA:

1. Mix the dC tailing mix as described in app. I table 11.

App. I. table 11. dC tailing mix

Components	**Volume (µl)**
DEPC-treated water	6.5
5 x tailing buffer	5.0
2 mM dCTP	2.5
S.N.A.P.-purified cDNA	10.0
Final volume	**24.0 µl**

2. Incubate the dC tailing mix at 94°C for 3 minutes. Chill on ice for 1 minute.

3. Add 1 µl terminal deoxynucleotidyl transferase (TdT), mix gently.

4. Run the following program on a thermal cycler: 37°C for 10 minutes followed by 65°C for 10 minutes. Collect the content by briefly centrifugation and place on ice (step 3, app. I. Fig. 1).

5. This is a safe stopping point. Store the dC-tailed cDNA at -20°C or proceed to the next step.

PCR amplification of dC-tailed cDNA:

For PCR amplification of the dC-tailed cDNA the AccuPrime Taq DNA Polymerase High Fidelity Kit from Invitrogen was used. The reaction mixture was composed as listed in app. I. table 12.

App. I. table 12. Reaction mixture for PCR amplification of dC-tailed cDNA.

Components	**Volume (µl)**
RNAse free water	35.5
10X AccuPrime PCR buffer I	5.0
GSP2-538 (10 µM)	2.0
Abridge Anchor Primer (AAP) (10 µM)	2.0
AccuPrime Taq DNA polymerase	0.5
dC-tailed cDNA	5.0
Final volume	**50 µl**

The PCR amplification was carried out using the same thermal cycler program as for conventional PCR (app. I. table 8) (step 4, app. I. Fig. 1).

The PCR amplicons were analyzed by agarose gel electrophoresis (2 % E-gel). If multiple bands appeared on the gel, amplicons of 500-600 bp in size were cut from the gel and purified using the High Pure PCR Product Purification Kit (Roche) following the procedure described in the section ‘Purification of PCR amplicons’.

Nested PCR amplification:

The nested PCR amplification was carried out using the AccuPrime Taq DNA Polymerase High Fidelity Kit from Invitrogen. The reaction mixture was prepared as listed in app. I. table 13.

App. I. table 13. Reaction mixture for nested PCR amplification.

Components	**Volume (µl)**
RNAse free water	37.5
10X AccuPrime PCR buffer I	5.0
Nested-458 (10 µM)	1.0
AUAP (10 µl)	1.0
AccuPrime Taq DNA polymerase	0.5
Purified PCR amplicons from the first PCR reaction	5.0
Final volume	**50 µl**

The nested PCR amplification was carried out using the same thermal cycler program as for conventional PCR (app. I. table 8) (step 5, app. I. Fig. 1).

The PCR products were analyzed on a 2 % agarose gel and purified as previously described (section: 'Purification of PCR amplicons').

The nested PCR amplicons was approximately 500 bp in size and ready for cloning into the TOPO vector. This was performed using the TOPO TA Cloning Kit for sequencing from Invitrogen.

The TOPO Cloning Reaction:

1. Mix the TOPO Cloning reaction in the order listed in app. I. table 14. Incubate the mixture for 5 minutes at RT.

2. Place the reaction on ice and proceed to Transform One Shot TOP10 Competent cells.

App. I. table 14. The TOPO Cloning reaction mixture.

Reagents	**Volume (µl)**
Nested PCR amplicons	4
Salt Solution	1
TOPO vector	1
Final volume	**6 µl**

Transform One Shot TOP10 Competent Cells:

1. Add 2 µl of the TOPO Cloning reaction into a vial of thawed One Shot Chemically Competent *E. coli* and mix gently. Do not pipeting up and down!

2. Incubate on ice for 5 minutes.

3. Heat-shock the cells at 42°C for 30 seconds, then place on ice.

4. Add 250 µl of RT equilibrated S.O.C. medium.

5. Incubate the cells at 37°C for 1 hour.

6. From each Transformed cell vial 50, 100, and 250 µl cell suspension was spread onto separate 37°C pre-warmed LB plates containing 100 µg/ml ampicillin.

7. The plates incubate at 37°C over night.

8. Next day, single colonies are selected and spread onto new 37°C pre-warmed LB plates containing 100 µg/ml ampicillin. The plates incubate at 37°C over night.

9. The following day, single colonies are selected for colony PCR. One colony was used per PCR reaction.

10. For the colony PCR the AccuPrime Taq DNA Polymerase High Fidelity Kit from Invitrogen was used. The reaction mixture was prepared as listed in app. I. table 15.

App. I. table 15. PCR colony PCR Reaction mixture.

Reagents	**Volume (µl)**
RNAse free water	42.5
10X AccuPrime PCR buffer I	5.0
M13-Fw (10 µM)	1.0
M13-Rev(10 µM)	1.0
AccuPrime Taq DNA polymerase	0.5
One *E. coli* colony	-
Final volume	**50 µl**

11. The colony PCR was carried out using the same thermal cycler program as for conventional PCR (table 2.8). The elongation step was extended to 90 seconds.

12. The colony PCR was examined on a 2 % agarose gel with the expected size of ~680 bp (500 bp from the nested PCR amplification product + 2 x 90 bp for the M13 sequence). Plasmids from plates containing positive colonies were purified using the PureLink Quick Plasmid DNA Miniprep kit from Invitrogen.

Purification of plasmid DNA:

1. Positive colonies were dissolved in 250 ml Resuspension Buffer (R3) with RNAse A by vortexing until homogeneous.

2. 250 ml Lysis Buffer (L7) was added and gently mixed by inverting the tube five times. Do not vortex. Incubate at RT for 5 minutes.

3. 350 µl Precipitation Buffer (N4) was added and mix by immediately inverting the tube until homogeneous. Do not vortex. Centrifuge the lysate at 12,000 x g for 10 minutes.

4. Transfer the supernatant to the spin column. Centrifuge at 12,000 x g for 1 minute. Discard the flow-through.

5. 700 µl Wash Buffer (W9) with EtOH was added the column and centrifuged at 12,000 x g for 1 minute. The flow-through was discarded and the column was centrifuged an additional time.

6. The column was transferred to a 1.5 ml eppendorf tube and 75 µl 65°C pre-heated TE Buffer (TE) was added to the center of the column. Then incubate at RT for 1 minute.

7. Elute the plasmid DNA by centrifuge the column at 12,000 x g for 2 minutes.

8. The concentration of the plasmid DNA was determined using a spectrophotometer.

Cycle sequencing of plasmid DNA:

For cycle sequencing, 1 µg of purified plasmid DNA in 10 µl MQ was prepared with 4 µl 5 µM M13 primers and send to LGC Genomics GmbH (Berlin, Germany). The obtained raw data was assembled into contigs using the software CLC Main workbench v. 6.6.2 (CLC BIO, Aarhus, Denmark). The obtained 5'-end nucleotide sequences were assembled with the nucleotide sequences obtained from next generation sequencing to form complete PRRSV genomes.

APPENDIX II

App. II. table 1. All Type 1 PRRS viruses sequenced during the PhD project.

Type 1 PRRSV	Isolation year	Sequence material	ORF5	ORF7	ORF2-7	Complete genome
ESP-1991-Olot91	1991/Spain	Marc-145 > 3. passage	-	-	-	KC862570
DK-1992-PRRS-111_92	1992	PAM 17. passage	-	-	-	KC862566
DK-2003-6-2	2003	PAM 1. passage	KC862530	KC862559	-	-
DK-2003-6-3	2003	PAM 1. passage	KC862533	KC862560	-	-
DK-2003-6-5	2003	PAM 1. passage	-	-	-	KC862571
DK-2003-7-2	2003	PAM 2. passage	-	-	-	KC862572
DK-2003-8-2	2003	PAM 1. passage	KC862531	KC862557	-	-
DK-2003-8-3	2003	PAM 1. passage	KC862532	KC862558	-	-
DK-2007-10-1-1	2007	Lung homogenate	-	KC862554	-	-
DK-2007-10-1-3	1007	Lung homogenate	KC862528	KC862555	-	-
DK-2007-80-2-1	2007	PAM 1. passage	KC862529	KC862556	-	-
DK-2008-10-5-2	2008	Lung homogenate	-	-	-	KC862573
DK-2008-10-5-3	2008	Lung homogenate	KC862526	-	-	-
DK-2008-16-6-1	2008	Lung homogenate	KC862527	KC862553	-	-
DK-2010-10-9-1	2010	Nasal swab	-	KC862534	-	-
DK-2010-10-10-3	2010	PAM 1. passage	-	-	-	KC862568
DK-2010-30-11-11	2010	Lung homogenate	-	-	KC862562	-
DK-2010-30-11-14	2010	Lung homogenate	-	-	KC862563	-
DK-2010-30-11-17	2010	Lung homogenate	-	-	KC862561	-
DK-2010-10-12-1	2010	Lung homogenate	-	-	KC862564	-
DK-2011-05-11-3	2011	Serum	KC862504	KC862535	-	-
DK-2011-05-11-5	2011	Serum	KC862505	KC862536	-	-
DK-2011-05-11-14	2011	Serum	-	-	-	KC862567
DK-2011-05-11-19	2011	Serum	KC862506	KC862537	-	-
DK-2011-05-11-20	2011	Serum	KC862507	KC862538	-	-
DK-2011-05-23-2	2011	Serum	KC862508	KC862539	-	-
DK-2011-05-23-9	2011	PAM 1. passage	-	-	-	KC862569
DK-2011-05-25-6	2011	Serum	KC862509	KC862540	-	-
DK-2011-05-25-8	2011	Serum	KC862510	KC862541	-	-
DK-2011-05-25-17	2011	Serum	KC862511	KC862542	-	-
DK-2011-05-25-20	2011	Serum	KC862512	KC862543	-	-
DK-2011-30-7-1	2011	Nasal swabs	KC862513	KC862544	-	-
DK-2011-30-8-12	2011	Serum	KC862514	KC862545	-	-
DK-2011-30-9-13	2011	Serum	KC862515	KC862546	-	-
DK-2011-30-9-17	2011	Serum	KC862516	-	-	-
DK-2011-09-02-2	2011	Serum	KC862517	KC862547	-	-
DK-2012-01-05-2	2012	Serum	-	-	-	KC862574
DK-2012-01-05-11	2012	Serum	KC862518	KC862548	-	-
DK-2012-30-8-8	2012	Nasal swabs	KC862519	KC862549	-	-
DK-2012-10-9-3	2012	Oral swab	KC862520	-	-	-
DK-2012-10-01-27	2012	Serum	KC862521	-	-	-
DK-2012-08-21-32	2012	Serum	KC862522	-	-	-
DK-2013-10-1-1	2013	Nasal swabs	KC862523	KC862550	-	-
DK-2013-10-2-1	2013	Lung homogenate	KC862524	KC862551	-	-
DK-2013-30-3-6	2013	Serum	KC862525	KC862552	ORF6:	KC862565

APPENDIX III

App. III table 1. All Type 2 PRRS viruses sequenced during the PhD project.

Type 2 PRRSV	Isolation year	Sequence material	ORF5	ORF7	Partial NSP2	Complete genome
DK-1997-19407B	1997	Marc-145 2. passage	-	-	-	KC862576
DK-2003-1-2	2003	Marc-145 1. passage	KC506665	KC506718	KF311033	-
DK-2003-2-3	2003	Marc-145 1. passage	-	-	-	KC862584
DK-2003-3-3	2003	Marc-145 1. passage	KC506667	KC506720	KF311034	-
DK-2003-4-1	2003	-	KC506668	-	-	-
DK-2003-5-1	2003	-	KC506669	-	-	-
DK-2004-1-3-Lu	2004	Marc-145 1. passage	KC506661	KC506714	KF311035	-
DK-2004-1-4-Lu	2004	Marc-145 1. passage	KC506662	KC506715	KF311036	-
DK-2004-1-7-Pl	2004	Marc-145 1. passage	-	-	-	KC862578
DK-2004-2-1	2004	Marc-145 1. passage	-	-	-	KC862585
DK-2004-3-1	2004	-	KC577601	KC577602	-	-
DK-2008-10-1-1	2008	Lung homogenate	-	KC506721	-	-
DK-2008-10-1-2	2008	Lung homogenate	-	KC506722	-	
DK-2008-10-1-3	2008	Lung homogenate	-	-	-	KC862582
DK-2008-16-2-3	2008	Lung homogenate	-	KC506724		-
DK-2008-16-2-4	2008	Lung homogenate	KC506671	KC506725	KF311037	-
DK-2008-16-3-3	2008	Lung homogenate	KC506672	KC506726	-	-
DK-2008-16-4-2	2008	Lung homogenate	-	KC577603	-	-
DK-2010-10-1-1	2010	Lung homogenate	KC506625	KC506674	-	-
DK-2010-10-1-2	2010	Lung homogenate	-	-	-	KC862579
DK-2010-10-2-1	2010	Marc-145 2. passage	-	-	-	KC862581
DK-2010-10-2-2	2010	Lung homogenate	KC506628	KC506677	KF311038	-
DK-2010-10-2-3	2010	Lung homogenate	KC506629	KC506678	KF311039	-
DK-2010-10-3-3	2010	Lung homogenate	KC506630	KC506679	KF311040	-
DK-2010-10-4-1	2010	Lung homogenate	-	-	-	KC862583
DK-2010-10-4-2	2010	Lung homogenate	KC506632	KC506681	KF311041	-
DK-2010-10-4-3	2010	Lung homogenate	KC506633	KC506682	KF311042	-
DK-2010-10-5-2	2010	Lung homogenate	KC506634	KC506683	KF311044	-
DK-2010-10-6-3	2010	Lung homogenate	KC506635	KC506684	-	-
DK-2010-10-7-1	2010	Marc-145 2. passage	-	-	-	KC862580
DK-2010-30-8-4	2010	Serum	KC506637	KC506686	-	-
DK-2010-10-13-1	2010	Marc-145 2. passage	-	-	-	KF183946
DK-2010-10-13-2	2010	Lung homogenate	KF183948	KF183961	KF311020	-
DK-2010-10-13-3	2010	Lung homogenate	KF183949	KF183962	KF311021	-
DK-2010-10-13-4	2010	Lung homogenate	KF183950	KF183963	KF311022	-
DK-2010-10-13-5	2010	Lung homogenate	KF183951	KF183964	KF311023	-
DK-2010-10-13-6	2010	Lung homogenate	KF183952	KF183965	KF311024	-
DK-2010-10-13-7	2010	Lung homogenate	KF183953	KF183966	KF311025	-
DK-2010-10-13-8	2010	Lung homogenate	KF183954	KF183967	KF311026	-
DK-2010-10-13-9	2010	Lung homogenate	KF183955	KF183968	KF311027	-
DK-2011-88005-A5	2011	Lung homogenate	KF183956	KF183969	KF311028	-
DK-2011-88005-A6	2011	Lung homogenate	KF183957	KF183970	KF311029	-
DK-2011-88005-A7	2011	Lung homogenate	KF183958	KF183971	KF311030	-
DK-2011-88005-A8	2011	Lung homogenate	KF183959	KF183972	KF311031	-
DK-2011-88005-A8-Pl	2011	Pleura	-	-	-	KF183947
DK-2011-88005-C1	2011	Lung homogenate	KF183960	-	KF311032	-
DK-2011-30-1-31	2011	Serum	KC506638	KC506687	KF311045	-
DK-2011-30-1-34	2011	Serum	KC506639	KC506688	KF311046	-
DK-2011-30-1-35	2011	Serum	KC506640	KC506689	KF311047	-
DK-2011-10-2-1	2011	Lung homogenate	KC506641	KC506690	-	-

Type 2 PRRSV	Isolation year	Sequence material	ORF5	ORF7	Partial NSP2	Complete genome
DK-2011-030311-1	2011	Lung homogenate	-	-	-	KC862577
DK-2011-030311-2	2011	Lung homogenate	KC506643	KC506692	KF311055	-
DK-2011-030311-3	2011	Lung homogenate	KC506644	KC506693	KF311056	-
DK-2011-030311-4	2011	Lung homogenate	KC506645	KC506694	KF311057	-
DK-2011-30-3-13	2011	Serum	KC506646	KC506695	KF311052	-
DK-2011-30-3-15	2011	Serum	KC506647	KC506696	KF311053	-
DK-2011-30-3-20	2011	Serum	KC506648	KC506697	KF311048	-
DK-2011-30-4-3	2011	Serum	KC506649	KC506698	KF311049	-
DK-2011-30-4-5	2011	Serum	KC506650	KC506699	KF311050	-
DK-2011-30-4-7	2011	Serum	KC506651	KC506700	KF311051	-
DK-2011-10-5-1	2011	Lung homogenate	KC506652	KC506701	KF311043	-
DK-2011-30-6-27	2011	Serum	KC506653	KC506702	KF311054	-
DK-2012-10-1-5	2012	Nasal swab	KC506654	KC506703	-	-
DK-2012-10-2-8	2012	Oral swab	KC506655	KC506704	-	-
DK-2012-10-2-9	2012	Oral swab	KC506656	KC506705	-	-
DK-2012-10-3-1	2012	Oral swab	KC506657	KC506706	-	-
DK-2012-10-4-2	2012	Nasal swab	-	KC506707	-	-
DK-2012-10-5-3	2012	Oral swab	-	KC506708	-	-
DK-2012-10-5-4	2012	Oral swab	-	KC506709	-	-
DK-2012-10-6-2	2012	Lung homogenate	-	KC506710	-	-
DK-2012-30-7-16	2012	Serum	KC506658	KC506711	-	-
DK-2012-30-7-18	2012	Serum	KC506659	KC506712	-	-
DK-2012-01-11-3	2012	Serum	-	-	-	KC862575