Laboratory of Virology

Department of Virology, Parasitology and Immunology

Faculty of Veterinary Medicine

Ghent University

ADAPTABLE INACTIVATED VACCINE FOR COMBATING PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Marc Geldhof

Thesis for obtaining the degree of Doctor in Veterinary Sciences (PhD), 2013

Promoters:

Prof. dr. Hans J. Nauwynck

Dr. Wander Van Breedam

Dutch translation of the title:

Aanpasbaar geïnactiveerd vaccin voor de bestrijding van het Porcien Reproductief en Respiratoir Syndroom Virus

Geldhof M. (2013) Adaptable inactivated vaccine for combating Porcine Reproductive and Respiratory Syndrome Virus. Dissertation, Ghent University, Belgium

ISBN 978-9-05864-354-4

The Federal Public Service Health, Food Chain Safety and Environment of the Belgian Federal Government (Convention RT -Thema 7 – Acron. PORRS) funded Marc Geldhof.

COVER: 3D Archigraphs Creative pigs

A couple of hundred years ago, Benjamin Franklin shared with the world the secret of his success. Never leave that till tomorrow, he said, which you can do today. You think more people would listen to what he had to say. I don't know why we put things off, but if I had to guess, I'd have to say it has a lot to do with fear. Fear of failure, fear of rejection, sometimes the fear is just of making a decision, because what if you're wrong? What if you're making a mistake you can't undo? He who hesitates is lost. We can't pretend we hadn't been told. We've all heard the proverbs, heard the philosophers, heard our (grand-) parents warning us about wasted time and heard the damn poets urging us to seize the day. Still sometimes we have to see for ourselves. We have to make our own mistakes. We have to learn our own lessons. We have to sweep today's possibility under tomorrow's rug until we can't anymore. Until we finally understand for ourselves what Benjamin Franklin really meant. That knowing is better than wondering, that waking is better than sleeping, and even the biggest failure, even the worst, beat the hell out of never trying.

M. Grey

TABLE OF CONTENTS

List of Abbreviations

LIST OF ABBREVIATIONS

aa	amino acid
ADCC	antibody-dependent, cell-mediated cytotoxicity
ADCML	antibody-dependent, complement-mediated lysis
ADEI	antibody-dependent, enhancement of infection
AEC	3-amino-9-ethylcarbazole
BAL	bronchoalveolar lavage
BEI	binary ethylenimine
cDNA	complementary DNA
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
DNA	desoxyribonucleic acid
dpi	days post inoculation
ds	double-stranded
E	small envelope protein
EAV	equine arteritis virus
ER	endoplasmic reticulum
ELISA	enzyme-linked immunosorbent assay
EU type	European type
FCS	fetal calf serum
GP	glycoprotein
HBV	hepatitis B virus
hpi	hours post inoculation
HS	heparan sulphate
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPMA	immunoperoxidase monolayer assay
Kb	kilobase

kDa	kilodalton
KV	killed virus
LDV	lactate dehydrogenase-elevating virus
LV	Lelystad virus
M	matrix protein
mAb	monoclonal antibody
MEM	Minimum Essential Medium
MHC	major histocompatibility complex
MLV	modified live virus
mRNA	messenger RNA
MSD	mystery swine disease
Mw	molecular weight
N	nucleocapsid protein
NA type	North-American type
NK	natural killer
nsp	non-structural protein
o/w	oil-in-water
ORF	open reading frame
PAM	porcine alveolar macrophages
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PEPscan	peptide scanning
PFU	plaque forming unit
pi	post inoculation
PK-15	porcine kidney cell line
PRDC	porcine respiratory disease complex
PRRS	porcine reproductive and respiratory syndrome

PRRSV	porcine reproductive and respiratory syndrome virus
PRV	pseudorabies virus
RNA	ribonucleic acid
RPMI	roswell park memorial institute medium
SHFV	simian hemorrhagic fever virus
Sn	sialoadhesin
SN	seroneutralization
SPF	specific pathogen free
ss	single-stranded
ST	swine testicle cells
$TCID_{50}$	tissue culture infectious dose with a 50 % end point
TGEV	transmissible gastroenteritis virus
TGFβ	transforming growth factor β
Th lymphocyte	T helper lymphocyte
TNF	tumor necrosis factor
Treg	regulatory T-lymphocyte
UV	ultraviolet
VN	virus-neutralizing
VZV	Varicella-zoster virus
WB	Western blot
WT type	wild type

CHAPTER 1

INTRODUCTION

1.1. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

1.1.1. HISTORY

A new pig disease causing reproductive failure in sows and severe pneumonia in piglets was first reported in the United States in 1987 and Canada in 1988 (Hill, 1990; Keffaber, 1989), and Western Europe in 1990 (Wensvoort et al., 1991). The disease was characterized by a high frequency of late-term abortions and an increased number of mummified, stillborn and weak-born piglets and by reduced conception rates in gilts and sows. Clinical signs observed in pigs were coughing, dyspnea, decreased feed efficiency, anorexia, growth retardation, increased mortality in pigs between 16 and 22 weeks of age and mild flu-like signs in nursery, growing, and finishing pigs (Christianson et al., 1992; Collins et al., 1992; Keffaber, 1989; Rossow, 1998, Zimmerman et al., 2006) and in some affected animals a red-blue discoloration of the ears was observed. In the beginning, the disease was named 'porcine epidemic abortion and respiratory syndrome' or 'swine infertility and respiratory syndrome' or 'mystery swine disease' or 'blue-eared pig disease' reflecting some of the characteristic symptoms. Later on, the disease was called worldwide 'porcine reproductive and respiratory syndrome' or PRRS (Christianson et al., 1992; Collins et al., 1992; Wensvoort et al., 1991). In 1991, researchers from Lelystad (The Netherlands) succeeded to isolate a virus from affected animals in primary alveolar macrophages, which they called 'Lelystad virus', and the clinical picture of PRRS could be experimentally reproduced by inoculation of pregnant sows with this virus (Terpstra et al., 1991; Wensvoort et al., 1991). Around the same time, American scientists were able to grow an American isolate of the virus in CL-2621 cells and confirmed its relation with the disease by experimental reproduction and named this isolate VR-2332 (Benfield et al., 1992; Collins et al., 1992). In the years following the first isolation of porcine reproductive and respiratory syndrome virus (PRRSV), the virus was spreading around the world, not only in Europe and the United States, but also in Canada and Asian countries (Baron et al., 1992; Dea et al., 1992; Hopper et al., 1992; Jiang et al., 2000; Plana et al., 1992; Shimizu et al., 1994). The origin of PRRSV and how the virus emerged independently on two different continents is still a matter of debate. At present, PRRSV is endemic in swine-producing countries worldwide (Albina, 1997; Cho & Dee, 2006). Phylogenetic studies have shown that

two PRRSV genotypes exist: the European (EU) genotype, with Lelystad virus (LV) as prototype EU PRRSV strain; and the North American (NA) genotype, with VR-2332 as prototype NA PRRSV strain (Collins et al., 1992; Nelsen et al., 1999; Snijder et al., 2004; Wensvoort et al., 1991). These genotypes are only far related (55 to 70 % nucleotide identity), but also within each genotype a high degree of genetic and antigenic variation is seen (Indik et al., 2000; Kapur et al., 1996; Meng et al., 1995; Morozov et al., 1995; Nelsen et al., 1999; Stadejek et al., 2002; Stadejek et al., 2006; Stadejek et al., 2008).

At the beginning of the PRRSV outbreaks, the EU genotype was restricted to Europe, and the NA genotype was restricted to America (Andreyev et al., 1997) and Asia (Shibata et al., 1996). However, the NA genotype appeared in Europe upon use of the RespPRRS vaccine, a modified-live vaccine based on VR-2332 (Bøtner, 1997; Madsen et al., 1998; van Vugt et al., 2001). In addition, EU genotype isolates appeared in North America upon import of European pigs (Dewey et al., 2000). Consequently, both genotypes now co-exist in several European, American and Asian swine-producing countries (Ropp et al., 2004). PRRSV appears to be continuously evolving (Chang et al., 2002; Goldberg et al., 2003; Rowland et al., 1999b), which results in the emergence of new PRRSV variants. The pathogenicity of different virus variants can vary greatly. While many PRRSV isolates have been described as apathogenic or only moderately virulent, different studies report on the emergence of higly pathogenic PRRSV variants (Bøtner et al., 1997; Epperson, 1997; Karniychuk et al., 2010; Mengeling et al., 1998; Tian et al., 2007; Tong et al., 2007; Zhou et al., 2008). For example, China and surrounding countries have suffered from an extremely severe epidemic that was attributed to a highly virulent PRRSV strain (HP-PRRSV) (Normile, 2007; Tian et al., 2007). To date, the factors that determine the virulence of PRRSV remain poorly understood. Further studies on the PRRSV biology, including pathogenesis, immunology and epidemiology are necessary to gain better insights into the evolution of this pathogen and its interaction with the host. Despite the availability of several attenuated and inactivated PRRSV vaccines and the great efforts of pig holders, veterinarians and researchers, the disease remains difficult to control (Bøtner et al., 1997; Cano et al., 2007; Epperson, 1997; Labarque et al., 2004; Prieto et al., 2008; Tian et al., 2007; Tong et al., 2007; Zhou et al., 2008) and imposes a substantial burden on swine producers with an estimated annual cost of 560

million dollars in the USA (Neumann et al., 2005; Pejsak et al., 1997). The real economical impact of the disease is however difficult to assess, since PRRS-related problems are not always directly visible, and often involve other viral and bacterial pathogens (Neumann et al., 2005; Thacker, 2001). Nevertheless, researchers, veterinarians and pig farmers worldwide consider PRRS as a major, if not the most important disease affecting the swine industry.

1.1.2. TAXONOMY

PRRSV belongs to the family of *Arteriviridae* (genus *Arterivirus*), which is grouped together with the *Coronaviridae* (genus *Coronavirus* and genus *Torovirus*) and *Roniviridae* (genus *Okavirus*) in the order of the *Nidovirales* (Sidell & Snijder, 2008). This classification was based on genome organisation and the relatedness of non-structural proteins used in RNA replication and transcription. The order name is derived from "*nidus*", latin for "nest", and refers to the nested set of 3' co-terminal subgenomic-length mRNAs that is generated for expression of the open reading frames (ORFs) downstream of the replicase gene (Snijder & Meulenberg, 1998). The vast majority of nidoviruses known to date can be assigned to one of these families. In addition to PRRSV, the *Arterivirus* family consists of the 'lactate-dehydrogenase elevating virus' (LDV) of mice, the 'equine arteritis virus' (EAV) of horses and the 'simian hemorrhagic fever virus' (SHFV) of certain monkey species (Conzelmann et al., 1993). Although they are derived from a common ancestor and share properties in genome organization, the members of the different *Nidovirus* families are quite different in certain features, including virion morphology, number and composition of structural proteins, the host, epidemiology, clinical disease, pathogenesis, and mechanism of persistent infection (Gorbalenya et al., 2006; Wills et al., 1997). *Arteriviruses* are enveloped single-stranded (ss) RNA viruses with a genome length of 13-16 kilobases (kb) and a diameter of 40-60 nm (Gorbalenya et al., 2006). The envelope contains 3 to 6 structural proteins, but in contrast to *Coronaviruses*, large spikes are absent from the *Arterivirus* surface (Snijder & Meulenberg, 1998). For all *Arteriviruses*, macrophages and/or endothelial cells are either the exclusive or the major target cells *in vivo*. Another common characteristic for *Arteriviruses* is the ability to establish persistent infections in their respective hosts (Gravell et al., 1986; Onyekaba et al, 1989; Timoney & McCollum, 1993; Wills et al., 1997). Phylogenetic

analysis based on the most conserved regions in 'open reading frame' 1b (ORF1b) indicates that PRRSV is most closely related to LDV, but the estimated divergence time of PRRSV and LDV from a common ancestor is still a matter of debate (Chen & Plagemann, 1995; Forsberg, 2005; Hanada et al., 2005).

1.1.3. STRUCTURAL BIOLOGY

Electron microscopical examination of purified extracellular PRRSV reveals that PRRS virions are pleiomorphic, but mostly spherical with a diameter of 40 to 60 nm (Benfield et al., 1992; Mardassi et al., 1994; Wensvoort et al., 1992). PRRS virions contain a nucleocapsid core of 25 to 35 nm, surrounded by a lipid bilayered envelope (Benfield et al., 1992; Mardassi et al., 1994; Wensvoort et al., 1992; Dokland, 2010). PH and temperature are 2 major factors that determine the stability of PRRSV (Bloemraad et al., 1994; Benfield et al., 1992). PRRSV infectivity rapidly decreases at 37 °C or 56 °C, but remains unchanged for 1 month at 4 °C. At lower temperatures (-20 °C; -70 °C), PRRSV is stable for months, when the pH of the culture medium is approximately 6.0 to 7.5. The virus is sensitive to acidic and alkalic pH, and because it is enveloped, it is not resistant to lipid solvents and chloroform (Benfield et al., 1992; Bloemraad et al., 1994). The complete genomes of several PRRSV strains, including LV and VR-2332, have been sequenced (Allende et al., 1999; Conzelmann et al., 1993; Gao et al., 2004; Meng et al., 1994; Meulenberg et al., 1993; Nelsen et al., 1999). The PRRSV genome is 15.1 kb in length for EU PRRSV strains and 15.4 kb for NA PRRSV strains (Allende et al., 1999; Meulenberg et al., 1993; Nelsen et al., 1999). The PRRSV genome consists of a positive single-stranded RNA molecule that contains 9 open reading frames (ORFs) and is expressed through a 3' coterminal nested set of polycistronic mRNAs with a common leader sequence at the 5' end.

The ORF1a and ORF1b genes encode two polyproteins that are posttranslationally processed into 14 non-structural proteins, while ORF2-ORF7 encode respectively the structural proteins GP2a, E, GP3, GP4, GP5, M and N. The structural ORFs are partially overlapping, and some parts of different proteins are therefore encoded by the same gene sequences. Furthermore, ORF2 encodes two complete structural proteins: GP2, encoded by ORF2a, and E, encoded by ORF2b, which is entirely embedded in ORF2a (Conzelmann et al., 1993; Meulenberg et al., 1993). For a comprehensive description of PRRSV genome organisation, transcription and

replication, readers should be referred to Gorbalenya et al. (2006).

The nucleocapsid of PRRSV is composed of nucleocapsid proteins (N), encoded by ORF7. The nucleocapsid was initially supposed to have an icosahedral shape, but a recent cryo-electron tomographic analysis indicated that the viral capsid does not show a clear isometric structure. More probably, dimers of the N protein are organized in a roughly helical organization around the viral RNA and interact with it via the N-terminal RNA-binding domain (Dokland, 2010; Spilman et al., 2009). The PRRSV envelope surrounds the nucleocapsid and contains six structural proteins: the unglycosylated proteins M and E and the glycoproteins GP2, GP3, GP4 and GP5, that all bear complex-type N-linked glycans.

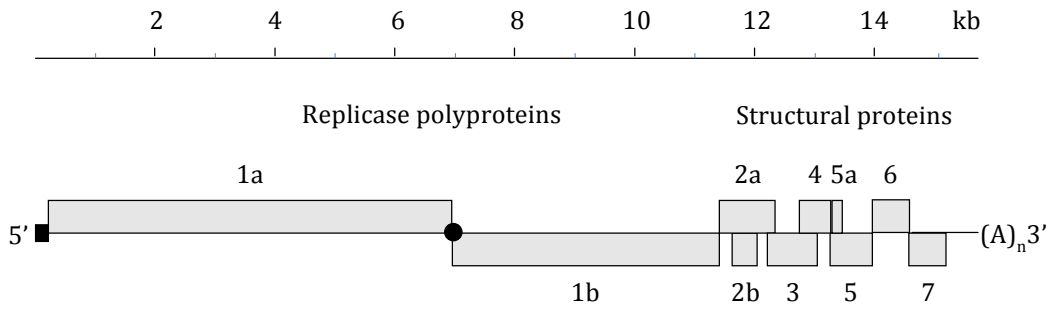


Figure 1. Schematic representation of the PRRSV genome organization (PhD Debaere M., 2012). The scalebar shows the length of the sequence in kb. Numbered grey colored blocks represent the different ORFs (the ORF encoding the recently discovered structural ORF5a protein is not depicted). The black box at the 5' end represents the leader sequence. The 3' end of the genome carries a poly A sequence (A). The black circle in the ORF1a/ORF1b overlap region indicates the site where the ribosomal frameshift occurs during translation of the ORF1ab polyprotein pp1ab.

GP5 and M are present in the virion as disulphide-linked heterodimers and are considered 'major' envelope proteins, while the remaining envelope proteins are most likely present in much lower amounts and are generally designated as 'minor' proteins (Meulenberg & Petersen-den Besten, 1996; Meulenberg et al., 1995; van Nieuwstadt et al., 1996; Wu et al., 2005). In contrast to M and GP5, the minor proteins are probably not essential for virion assembly, but the entire set of envelope proteins needs to be incorporated to render virus particles infectious. Furthermore, GP2, GP3 and GP4 need to be expressed simultaneously for incorporation in the virion, which indicates that these proteins interact with each other. However, in contrast to other *Arteriviruses*, there is no evidence for the existence of disulphide linkages between

the minor proteins of PRRSV (Wieringa et al., 2003; Wissink et al., 2005). Only very recently, Johnson and coworkers (2011) identified a novel structural PRRSV protein encoded by ORF5a (ORF5a protein) (Johnson et al., 2011). It cannot be excluded that future research leads to the identification of additional ORFs that encode important (non-) structural PRRSV proteins.

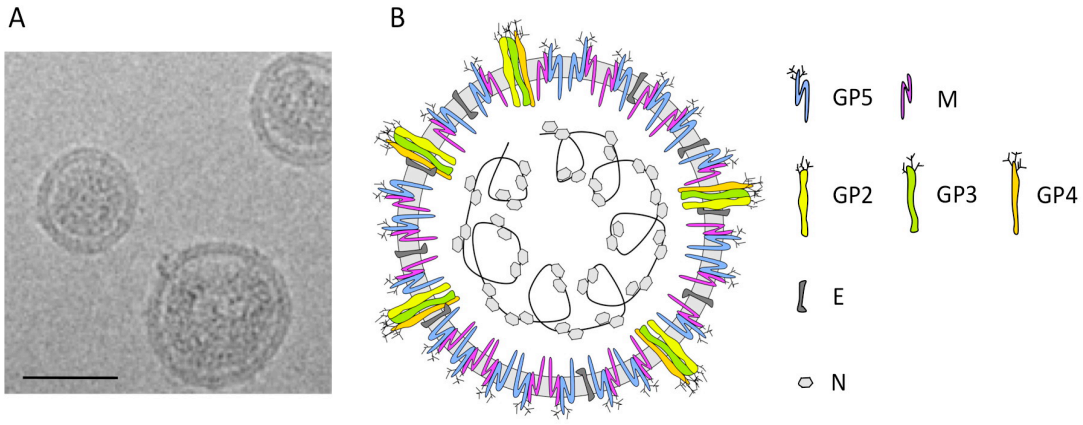


Figure 2. PRRS virion structure and morphology (PhD Van Gorp H., 2010).
(A) Cryo-electron microscopy of PRRSV particles (Spilman et al., 2009). Bar, 50nm. (B) Schematic representation of a PRRSV particle. PRRSV is composed of a nucleocapsid that is surrounded by an envelope in which viral proteins are embedded. The major membrane proteins are the glycoprotein GP5 and the membrane protein M, which are present as heterodimers. The minor membrane proteins are the envelope protein E and the glycoproteins GP2, GP3, and GP4, which are present as heterotrimeric complexes. The recently discovered structural ORF5a protein is not depicted.

1.1.4. HETEROGENEITY

PRRSV appeared 20 years ago almost simultaneously in Europe and the US, but it became rapidly clear that the virus showed remarkable genetic variation with two geographically distinct genotypes at the time of its discovery, indicating the possibility of prolonged evolutionary divergence prior to its appearance as a swine pathogen (Murtaugh et al., 2010). Therefore, PRRSV strains are classified into a European genotype (EU type or type 1) and a North American genotype (NA type or type 2), with the EU prototype LV and the NA prototype VR-2332 differing more than 40% at amino acid level (Collins et al., 1992; Nelsen et al., 1999; Wensvoort et al., 1991). In the nineties, Stadejek et al., (2002) demonstrated that the EU type PRRSV isolates that were circulating in Europe were extremely diverse but were all belonging to one subtype (subtype 1). However, when PRRSV isolates from countries of the former Soviet Union were sequenced, it became clear that these type 1 strains were more distant viruses (subtypes 2 and 3) (Stadejek et al., 2006). In time, both EU

type and NA type spread geographically, diverged genetically, and acquired new phenotypic characteristics, especially increased virulence. NA type PRRSV strains are frequently isolated in Europe nowadays and vice versa, and both genotypes are circulating in Asia as well (Murtaugh et al., 2010; Nielsen et al., 2001; Ropp et al., 2004; Shi et al., 2010). In Asia, most of the PRRSV isolates identified are of the NA type (Cha et al., 2006; Chueh et al., 1998; Jiang et al., 2000; Yoshii et al., 2005), with the exception of China and Thailand where EU type PRRSV was observed (Chen et al., 2011; Thanawongnuwech et al., 2004).

Figure 3. A phylogenetic tree of circulating virus isolates in Belgium with the prototype EU strain LV, the prototype NA strain VR-2332 and vaccine strains Porcilis® PRRS, and Ingelvac® PRRS MLV based on the N amino acid sequences. The neighbouring-joining method was used and numbers indicate bootstrap values of 100 replicates. Nomenclature of the PRRSV-positive isolates was mostly as follows: Year of isolation, Diagnostic number, Age of the animal if PRRSV-positive sample; e.g. 08V120-7w8 (Geldhof et al., 2013).

PRRSV is also largely prevalent in pig farms in Flanders, both in breeding animals and in fattening pigs (Geldhof, unpublished data). Virus isolates could be clearly grouped into EU type strains and NA type strains. It was shown that all NA type isolates were highly related to VR-2332, the strain on which the Ingelvac® PRRS MLV vaccine is based. However, it is not known whether the vaccine-like viruses that were isolated in this study were indeed underlying the problems that were observed at the respective vaccinating farms. NA-type virus was detected in 5 of the 19 herds. While animals in 3 of these herds were vaccinated with the corresponding vaccine, this vaccine had not been used in the remaining 2 herds. Prior use of this vaccine in these herds is unknown. Amongst the EU type strains that were isolated in this study, a considerable diversity was observed, and none of the isolates highly resembled the prototype EU strain LV, on which the Porcilis® PRRS and Progressis® vaccines are based. Furthermore, very closely related virus strains were isolated from the same

farm, indicating that they could have arisen from a common virus that had earlier been introduced on the farm. In contrast, it was demonstrated that different virus variants circulate within a single farm. Here, the two different PRRSV strains were probably introduced in the farm by two independent events, rather than being derived from one ancestral strain that was circulating in the herd. Surprisingly, similar virus strains were prevalent at geographically separated farms. It is currently not clear from where these isolates originated, and how they were independently introduced on two different farms. Taken together, it is clear that a large variability exists amongst PRRSV field strains that circulate on vaccinating farms in Flanders.

Phylogenetic analysis of PRRSV has provided a broadly applicable means to relate diverse isolates, but it does not explain biological variation in virulence or immunological cross-protection. Sequencing is best used to show the relatedness of strains over time and within a herd. Computer programs compare all possible pairs of sequences and phylogenetic trees or dendrograms can be constructed. Thus, at present both genotypes are spread across the world, where they keep on evolving with a mutation rate that is similar or even higher compared to other RNA viruses (Hanada et al., 2005; Prieto et al., 2009). The impact of vaccine usage on PRRSV evolution is at present unknown and perhaps the most significant long-term impact on PRRSV diversity and evolution will come from management changes preventing PRRS (Murtaugh et al., 2010).

1.1.5. PATHOGENESIS

Virus is shed in saliva (Wills et al., 1997), nasal discharge (Rossow et al., 1994), urine (Wills et al., 1997), semen or artificial insemination (Prieto et al., 1997; Swenson et al., 1994; Zimmerman et al., 2006) and feces (Christianson et al., 1993) and may cause horizontal PRRSV transmission between animals. Experimental infection can be achieved by intranasal, intratracheal, oronasal, oral, intramuscular, intra-uterine, intraveneous, intraperitoneal or vaginal inoculation (Albina et al., 1997; Benfield et al., 2000; Christianson et al., 1992; Christianson et al., 1993; Collins et al., 1992; Nodelijk et al., 2003; Rossow et al., 1994; Swenson et al., 1994; Van Reeth et al., 1999; Yaeger et al., 1993; Yoon et al., 1999). In the field, pigs are also susceptible to parenteral exposure routes by husbandry practices (ear notching, tail docking, teeth clipping…) or by normal pig behavior (bites, cuts, scrapes, tail-biting...). Airborne

transport of PRRSV has been documented to occur from 4.7-9.2 km, and is dependent on the PRRSV strain used (Dee et al., 2009; Otake et al., 2010). Aerosol transmission is not an equal component of area spread across NA type and EU type strains. Farms located nearby sites where PRRSV is prevalent may be at risk for a PRRSV airborne transmission. Air filtration is an effective tool to reduce the risk of external PRRSV introduction to breeding herds located in swine dense regions. Studies are currently underway to continue to assess the sustainability of air filtration and to calculate its cost/benefit (Dee et al., 2010; Linhares et al., 2012). Other ways of indirect transmission involves transmission by inanimate items (clothing, boots, equipment…) (Otake et al., 2002a), substances (water, food) and living carriers (vectors) (Otake et al., 2002b). In addition to horizontal spread, vertical transmission also occurs by viremic dams spreading PRRSV transplacentally to fetuses, resulting in fetal death or birth of infected pigs that are weak or appear to be normal (Christianson et al., 1992; Terpstra et al., 1991). The pathogenesis of PRRSV infection is based on the replication of PRRSV in cells of the monocyte/macrophage lineage (Duan et al., 1997a, b; Wensvoort et al., 1991). After uptake by the host, PRRSV replicates in the macrophage subsets of the respiratory tract, draining lymph nodes and tonsils, and arrives subsequently in the blood (Beyer et al., 2000; Paton & Drew, 1995; Rossow et al., 1995; Rossow et al., 1996). The specific tropism can be explained by the elegant interplay between viral and macrophage molecules (reviewed by Van Breedam et al., 2010). Upon PRRSV infection, there is an acute phase characterized by a high level of viremia, followed by a subacute phase with a low level of viremia. The viremia, which is not cell-associated, can be detected within 12 hpi to 3 dpi and can last for 28-42 dpi in suckling, weaned and grower pigs and for 7-14 dpi in sows and boars (Bilodeau et al., 1994; Christopher-Hennings et al., 1995a; Duan et al., 1997b; Paton & Drew, 1995; Rossow et al., 1995; Yoon et al., 1993). The blood virus titers reach a maximum at 5 to 14 dpi and then strongly decrease to lower levels (Christopher-Hennings et al., 1995a; Duan et al., 1997b; Labarque et al., 2000; Rossow et al., 1995). During viremia, PRRSV is distributed to macrophages in both lymphoid (lymph nodes, spleen, thymus) and non-lymphoid tissues (liver) (Beyer et al., 2000; Duan et al., 1997b; Halbur et al., 1995a; Lawson et al., 1997; Rossow et al., 1998). In boars, PRRSV is found in the testes and is shed in semen. The duration of semen shedding varies widely among boars (Christopher-Hennings et al., 1995a,b; Sur et al., 1997; Swenson et al., 1994). In pregnant sows, endometrium and placenta are major

sites of virus replication and at the end of gestation (> 80 days of gestation) the virus can cross the placenta and infect the fetuses. Transplacental spread of the virus during the third trimester of gestation may be explained by changes in the placenta during this stage of gestation (Christianson et al., 1992; Christianson et al., 1993; Karniychuk et al., 2011; Karniychuk et al., 2009; Rowland, 2010).

Although viremia is generally cleared within a few weeks, the virus can persist in lungs and lymphoid organs (tonsils, lymph nodes, spleen) and testes (Allende et al., 2000; Beyer et al., 2000; Duan et al., 1997a; Labarque et al., 2000; Wills et al., 1997; Wills et al., 2003). PRRSV persists in these organs through a low level of replication that may decrease over time (Allende et al., 2000). In some animals however, a viremia may recur (Christopher-Hennings et al., 1995a, b; Rowland & Yoo, 2003). The ability of PRRSV to cause persistent infections indicates that the virus is capable of evading the host defense mechanisms. Finally, most pigs resolve PRRSV infection within 2 to 4 months (Allende et al., 2000; Wills et al., 1997; Wills et al., 2003). The mechanisms responsible for the complete elimination of PRRSV also remain to be elucidated.

1.1.6. CLINICAL PICTURE

The clinical picture of PRRS in the field is quite variable regarding both the range of symptoms and severity. The outcome of a PRRSV infection is often influenced by other pathogens, environmental factors and farm management (Brockmeier et al., 2002). Furthermore, marked differences in virulence have been observed between different PRRSV isolates, both in the field and under controlled conditions (Epperson & Holler, 1997; Halbur et al., 1995; Halbur et al., 1996b; Mengeling et al., 1998; Rossow et al., 1999; Tian et al., 2007; Tong et al., 2007; Zhou et al., 2008), as exemplified by the severe PRRS-outbreaks in the USA around 1996, the emergence of highly pathogenic PRRSV in China in 2007, and the isolation of a highly virulent strain in Belarus (Halbur & Bush, 1997; Karniychuk et al., 2010; Tian et al., 2007). Nonetheless, PRRSV-associated disease mainly comprises respiratory distress in predominantly young animals and reproductive failure in sows of all parities.

PRRSV is one of the most frequently isolated pathogens from cases of ‘porcine respiratory disease complex’ (PRDC), and experimental co-infection studies have

demonstrated that the combination of PRRSV with several bacterial and viral pathogens results in a more severe clinical picture than either agent alone. Interactions between PRRSV and *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, and the combination of *Pasteurella multocida* with *Bordetella bronchiseptica* have been demonstrated (Brockmeier et al., 2002; Choi et al., 2003; Thacker, 2001). Furthermore, PRRSV was shown to enhance disease caused by porcine respiratory coronavirus and swine influenza virus (Van Reeth et al., 1996; Van Reeth et al., 2001). Clinical signs of PRDC are coughing, anorexia, growth retardation and increased mortality of piglets between 16 and 22 weeks old (Collins et al., 1992; Rossow 1998).

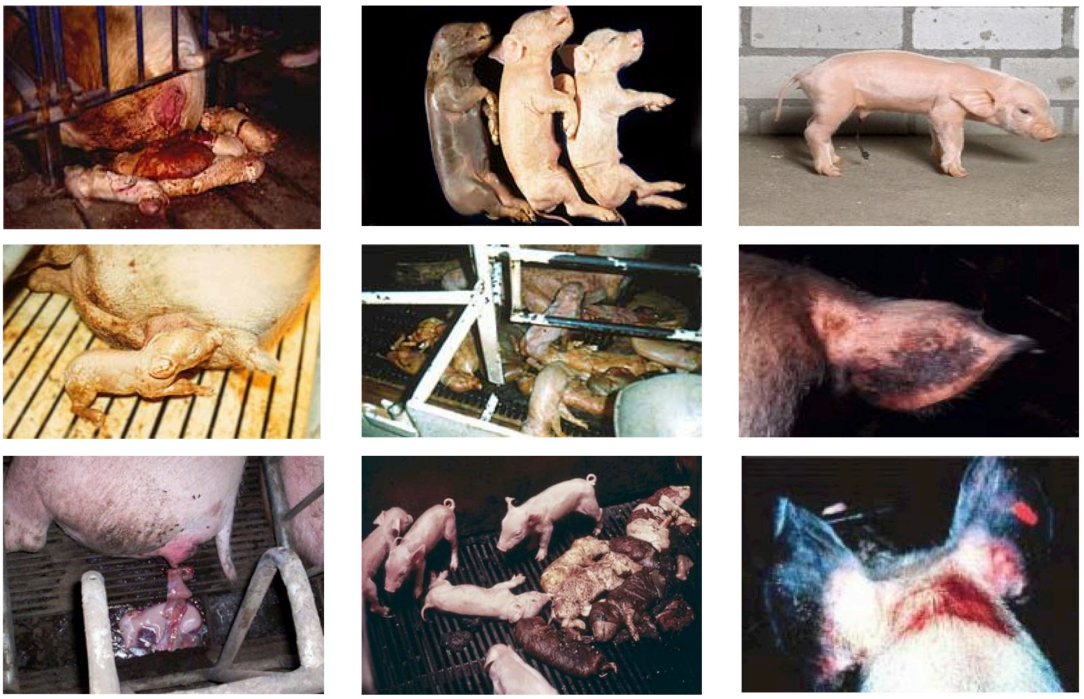


Figure 4. Clinical picture of PRRSV (adapted from www.respig.com; www.thepigsite.com; www.porcilis-prrs.com). Mummified, stillborn or weakborn piglets and a red-blue discoloration of the ears in affected animals.

PRRSV-associated reproductive failure generally takes place at the end of gestation and is characterized by late-term abortion or early farrowing, with a high number of mummified, stillborn or weakborn piglets. The incidence of PRRSV-induced abortion, transplacental spread and the birth of weak or dead piglets upon experimental infection is limited to the third trimester of gestation (Christianson et al., 1993; Christianson et al., 1992; Mengeling et al., 1994; Terpstra et al., 1991). Clinical signs in infected sows or gilts vary from none to anorexia, fever, lethargy and delayed

return to estrus and low conception rates on the subsequent breeding (Done and Patton, 1995; Hopper et al., 1992, Mengeling et al., 1994; Terpstra et al., 1991).

Intra-uterine virus inoculation at different time points revealed that fetuses are susceptible to PRRSV-infection throughout the entire gestation period, indicating that a barrier in transplacental spread is responsible for the limited time frame during which PRRSV-associated reproductive failure can occur (Lager & Mengeling, 1995). A possible explanation is that the number of susceptible cells in the uterus, placenta and fetal organs increases over time during pregnancy, as recently demonstrated by Karniychuk et al. (2010) and Karniychuk & Nauwynck (2009). However, the exact mechanisms of PRRSV-induced reproductive failure and transplacental spread remain unresolved at present.

In PRRSV-infected boars, during acute illness, in addition to anorexia, lethargy and respiratory clinical signs, the virus is found in the testes resulting in a temporary decrease of sperm quality (decrease in the sperm motility, spermatozoa with acrosomal defects) and shedding of the virus via sperm (Benfield et al., 1999; Christianson et al., 1994; Prieto & Castro, 2005), which may lead to virus transmission to sows.

1.2. PRRSV-INDUCED IMMUNE RESPONSE

The immune response is not efficient at all in controlling virus replication. The virus developed immune escape mechanisms at the level of both the innate and adaptive immune system. This is already visible at very early stages of infection. Nonetheless, infected-and-recovered pigs are generally well protected against re-infection with homologous virus, demonstrating that a protective immune response against PRRSV can indeed developed (Labarque et al., 2004; Labarque et al., 2003b; Lager et al., 1999; Mengeling et al., 2003; Nielsen et al., 1997). Despite many efforts, serious gaps still exist in our knowledge of (i) the events initiating the immune response during infection, (ii) key virological targets for both antibody and cell-mediated protection, (iii) the molecular and cellular mechanisms regulating induction and maturation of the immune responses, (iv) the consequences of genetic diversity in PRRSV on immune protection and (v) host genetic variation in pig populations on immune responsiveness to PRRSV. For a comprehensive description of PRRSV immunity, its

immunopathogenesis and immune evasion, readers are referred to Mateu and Diaz (2008) and Kimman et al. (2009). This section briefly summarizes what is known on PRRSV-specific immunity.

1.2.1. INNATE IMMUNITY

The innate immune system is an important arm of defense to prevent viral invasion and replication and to initiate the adaptive arm of the immune system. Adequate early activation of the innate immune system is critical to initiate generation of protective adaptive immunity to achieve complete viral clearance (Dwivedi et al., 2012). The innate immune system consists of **humoral** (cytokines, acute phase proteins and complement) and **cellular** components (natural killer cells, macrophages, dendritic cells, neutrophils and γδ T-lymphocytes). Type I interferons (IFN), including **IFNα** and **IFNβ**, are essential for the subsequent development of an effective adaptive immunity (reviewed in Takoaka & Yanai, 2006). They play a key role in antiviral immunity, by stimulating apoptosis in virus-infected cells, rendering cells resistant to viral infection, and driving many processes of the cell-mediated immune response (reviewed in Biron, 1998; reviewed in Fitzgerald-Bocarsly & Feng, 2007). Recently, Calzada-Nova, et al. (2011) found that NA type PRRSV isolates did not induce, or even strongly inhibited, IFN-α in plasmacytoid dendritic cells (pDC), representing "professional IFN-α-producing cells". Baumann et al. (2013) further characterized the PRRSV effects and host modifying factors on IFN-α responses of pDC and concluded that several types (of both EU and NA) stimulated IFN-α secretion by pDC suggesting that suppressive activities on pDC, if any, are moderate and strain-dependent. Besides type I IFN, **other cytokines** also contribute to the innate immune response to virus infections, such as TNFα/β, IL1, IL6, IL10, IL12, IL15 and IFNγ (reviewed in Kimman et al., 2009). For example: Interleukin-10 is involved in suppression of the T-helper-1 response, suppression of professional antigen-presenting cells and stimulation of B-cell proliferation and survival (Suradhat and Thanawongnuwegh, 2003) and TNF-α is involved in the induction of fever and the acute-phase response, and is able to stimulate macrophage activity (Gimeno et al., 2011). **Complement activation** results in an enzyme cascade, in which an activated component activates several molecules of a downstream component. **NK cells** produce TNFα/β and are the major producers of IFNγ during the early phase of infection (Borghetti, 2005; Lodoen

& Lanier, 2006; Newman & Riley, 2007; Pintaric et al., 2008). However, the direct effects of IFNγ on virus replication *in vivo* are unknown and the involvement of NK cells during PRRSV infection remains to be elucidated. Cao et al. (2012) showed that a PRRSV infection down-regulates the NK cell cytotoxicity. These results are in line with the study performed by Jung et al. (2009). Activated **macrophages** are important phagocytes, producing pro-inflammatory cytokines and possessing cytolytic activity (Janeway et al., 2005). Further, they represent an important connection between innate and adaptive immunity, since they process phagocytized antigen and present it to T-lymphocytes. Pigs possess a high proportion of circulating γδ **T-lymphocytes**: they can produce both T helper 1 cytokines (including IFNγ) and T helper 2 cytokines, fulfilling a regulatory function (Takamatsu et al., 2006). The role of γδ T-lymphocytes in the anti-PRRSV immune response remains to be elucidated. **Dendritic cells** recognize and process viral components and produce regulatory cytokines and leukocyte-attracting chemokines. Further, they transport processed viral antigens to the lymph nodes, where they present it to and activate virus-specific T-lymphocytes (Villadangos & Young, 2008). **Neutrophils** are normally found in the blood stream, but during the acute phase of inflammation, they migrate to the site of inflammation.

Although only incomplete, fragmented, and often contradicting data are available on the innate immune responses evoked by PRRSV, it can be stated that a strong innate immune response is absent upon a first PRRSV-infection.

1.2.2. ADAPTIVE IMMUNITY

Both PRRSV-specific cell-mediated and antibody-dependent immune mechanisms have been investigated, and some determinants of protective immunity have been described. However, it remains unclear to which extent different adaptive immune mechanisms contribute to protection against the virus. Like as for the PRRSV-induced innate immune response, scarce and often contradictory information is available on the PRRSV-specific immune response. Exact causes for the uncommon adaptive immune response to PRRSV are not yet known but it seems clear that the virus has developed mechanisms for escaping the immune system.

1.2.2.1. CELL-MEDIATED IMMUNITY

The cell-mediated immunity plays a critical role in the resolution of many virus infections, but is delayed for PRRSV in comparison with other viral infections (Bautista & Molitor, 1997). Proliferative T cell responses, mainly characterized by a type I cytokine expression phenotype of IFN-γ and IL-2, have been detected between 4 and 12 weeks after infection. However, the establishment of long-term persistence of the virus in the host suggests that cell-mediated immunity, including IFN-γ production, is not potent or ineffective in curtailing the infection (Batista et al., 2004; Murtaugh et al., 2002), although IFN-γ reduces PRRSV infection in porcine alveolar macrophages *in vitro*. IFN-γ secreting cells are mainly CD4+ CD8+ cells, with a few CD4−/CD8αβ+ cytotoxic T cells (Meier et al., 2003).

Upon virus infection, mature dendritic cells or other **antigen-presenting cells** present viral antigen in association with MHC-I or MHC-II molecules to respectively naïve CD8+ or CD4+ T-lymphocytes in the draining lymph nodes (Villadangos & Young, 2008). Activated CD8+ T-lymphocytes proliferate and differentiate into CD8+ cytotoxic T-lymphocytes (CTL) or CD8+ regulatory T-lymphocytes (Treg) (Bettelli et al., 2008; Roncarolo et al., 2006). At the place of infection, **CD8+ cytotoxic T-lymphocytes** generally locate virus-infected cells by screening MHC-I molecules on the cell surfaces and recognition of virus-infected cells results in elimination of these cells. Virus-specific $CD4^+CD8^-$ T cells can either represent **T-helper 1 (Th1) or T-helper 2 (Th2) cells**. The strong IL-10 response that is often observed favors a shift towards a Th2-response, although conflicting data exist about the Th1/Th2 balance upon PRRSV infection (Diaz et al., 2005; Lopez-Fuertes et al., 2000). The presence of functional CTL and the balance of CD4+ Th1 and Th2 lymphocytes during a viral infection are believed to determine the outcome of the infection. Virus-specific $CD4^-$ $CD8^+$ T-cells represent **CTL**, but Costers et al. (2009) demonstrated that these cells are not able to eliminate PRRSV-infected macrophages *in vitro* (Costers et al., 2009). This indicates that although PRRSV-specific CTL develop and infiltrate in the lungs, they are probably not able to clear infected lung macrophages. However, a not yet defined subpopulation of leukocytes is able to efficiently lyse PRRSV-infected macrophages (Costers et al, 2009). A transient decrease of $CD4^+$ and $CD8^+$ cells in blood is observed during the first days upon PRRSV-infection, together with an influx in the lungs of $CD8^+$ cells that initially consist of mainly **NK-like cells**. NK cells are

suppressed in their cytotoxic function (Renukaradhya et al, 2010). Finally, recent studies have demonstrated the potential of PRRSV to stimulate the development of **Tregs** *in vitro*, although the role of these cells *in vivo* remains to be elucidated (Silva-Campa et al., 2009; Wongyanin et al., 2010). Tregs have shown to be negative regulators *in vitro* by blocking lymphocyte proliferation, differentiation and effector functions, thereby preventing excessive immune responses (Roncarolo et al., 2006).

However, a live attenuated PRRSV vaccine that induces high IFN-γ -secreting cell frequencies protected pigs against viremia. Levels of IL-10 seemed to inversely correlate with interferon-γ responses. These results may indicate a strong involvement of T cell immunity, IFN-γ, and possibly of IL-10, in the development of immunity against PRRSV (Diaz et al., 2006). Increased levels of IL-10 in particular raise concerns that PRRSV is capable of shifting the immune response towards a less effective Th2-mediated immune response.

Activated T cells appear to be directed against the products of ORFs 2, 3, 4, 5, 6, and 7, but their protective role is unknown (Bautista et al., 1999). A couple of T-cell epitopes have been identified in GP4, GP5 and N of LV (Diaz et al., 2009). Vashisht et al. (2008) identified two distinct regions (amino acid residues 117–131 and 149–163) on GP5 of the NA genotype of PRRSV that appeared to contain immunodominant T-cell epitopes based on their ability to stimulate IFN-γ secreting cells. The sequence of most of these T-cell epitopes is largely conserved amongst the EU type field isolates, and for some also between EU- and NA type virus. Consequently, it is unknown how and to which extent conserved T cell epitopes are involved in PRRSV protective immunity. Still, for some proteins, T cell epitopes have not yet been identified at amino acid level. Molecular strategies may help to locate these epitopes. Methodological approaches are diverse and may include bio-informatic prediction, synthesis of peptides and *in vivo* testing.

1.2.2.2. HUMORAL IMMUNITY

1.2.2.2.1. ACTIVE HUMORAL IMMUNITY

The humoral immunity starts like with other viruses. PRRSV induces a robust **PRRSV-specific antibody response**, which can be detected by the following 5

serological tests: indirect fluorescent antibody (IFA), ELISA, blocking ELISA, serum-virus neutralization (SN), and immunoperoxidase monolayer assay (IPMA). Upon initial PRRSV infection, naïve animals seroconvert between 5 and 14 dpi and antibody titers increase rapidly to a maximum level around 4 weeks pi (Diaz et al., 2005; Meier et al., 2003; Yoon et al., 1995). Early antibodies seem to be mainly directed against the N protein and some non-structural proteins, while antibodies against the envelope proteins are usually detected at later time points (Oleksiewicz et al., 2001). Upon first infection, maximum antibody titers are generally 640 or 1240 with the IPMA, and a similar magnitude is obtained with the ELISA and IFA (Albina et al., 1998b; Batista et al., 2004; Diaz et al., 2005; Labarque et al., 2000; Vezina et al., 1996; Yoon et al., 1995). Anti-PRRSV IgM antibodies can be detected in serum starting from 6 to 8 dpi (Labarque et al., 2000; Loemba et al., 1996; Mulupuri et al., 2008), they peak at 8 to 14 dpi and disappear after 21 to 50 dpi (Labarque et al., 2000; Loemba et al., 1996; Mulupuri et al., 2008), whereas IgG (both IgG1 and IgG2) antibodies peak at 30 to 50 dpi and remain at high levels for months (Joo et al., 1997; Labarque et al., 2000; Loemba et al., 1996; Mulupuri et al., 2008; Vezina et al., 1996).

Antibodies can exert **antiviral activities** in different ways (Burton, 2002): antibodies can protect against viral infections, either by interacting with infectious virions and compromising their infectivity, or by binding viral antigens on infected cells, resulting in activation of the classical complement cascade or leading to lysis of these cells and thus prevention of further viral spread. Cell lysis as a result of virus-specific-antibody-binding, followed by complement activation is referred to as antibody-dependent, complement-mediated cell lysis (ADCML). In the latter case, infected cells that are opsonized by antibodies and killed by cytotoxicity exerted by immune cells with Fc receptors is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) (Burton, 2002; Janeway et al., 2005).

Antibodies against envelope proteins, amongst **PRRSV-neutralizing antibodies**, as determined by neutralization assays, usually do not increase before 3 to 4 weeks pi or do not appear at all (Delputte et al., 2004; Labarque et al., 2000; Loemba et al., 1996; Lopez & Osorio, 2004; Nelson et al., 1994). If they appear, they typically peak around 60 to 90 dpi and persist up to 1 year after infection at low levels (Albina et al., 1998b; Yoon et al., 1995). The reason why virus neutralizing (VN) antibodies appear so late in infection and remain at such low levels is not yet known. The high variability of the

virus, glycan shielding (Ansari et al., 2006; Darwich et al., 2010; Faaberg et al., 2006; Vu et al., 2011), decoy activities (Murtaugh et al., 2002; Ostrowski et al., 2002), interference with the innate immune response (Darwich et al., 2010; Flores-Mendoza et al., 2008), are forwarded as main reasons for the late appearance of VN antibodies.

The role of VN antibodies in the resolution of a PRRSV infection and protection remains unclear. VN antibodies generally appear within the time frame of viral clearance from lungs and blood, suggesting that they might be important. A correlation has been reported between the appearance of VN antibodies and clearance of cell-free virus from circulation / protection against viremia (Murtaugh et al., 2002; Pirzadeh & Dea, 1998). Moreover, it has been reported that a passive transfer of VN antibodies can protect pregnant sows against PRRSV-associated reproductive failure and transplacental spread, as well as against virus replication in tissues and viremia upon challenge with infectious virus (Osorio et al., 2002).

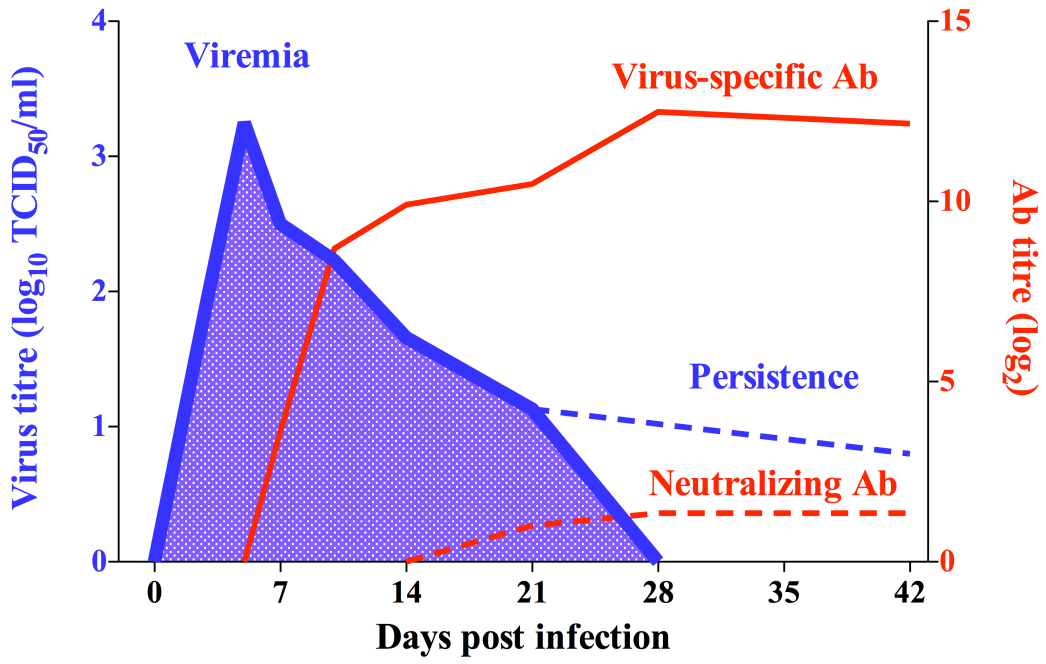


Figure 5. Schematic overview of viremia and the virus-specific and virus-neutralizing antibody response upon PRRSV infection in pigs (PhD Vanhee M., 2011).

Passive transfer of VN antibodies can also provide sterilizing immunity to the offspring, but titers ≥ 32 are needed to prevent viremia (Lopez et al., 2007). These findings suggest that VN antibodies can protect animals from PRRSV infection and

have the potential to eliminate free virus from circulation.

On the other hand, other studies do not support a strong correlation between the development of VN antibodies and the clearance of viremia (Diaz et al., 2006; Xiao et al., 2004; Zuckermann et al., 2007). Also, it was observed that PRRSV might persist in the lungs and lymphoid tissues of PRRSV-infected pigs, despite the presence of VN antibodies (Labarque et al., 2000; Lopez & Osorio, 2004; Murtaugh et al., 2002; Osorio et al., 2002; Wills et al., 1997).

Importantly however, the VN antibodies are to a large extent strain specific, much more than the non-neutralizing antibodies and a lack in cross-neutralization often occurs even between genetically closely related virus strains (Kim & Yoon, 2008; Okuda et al., 2008). The magnitude of the VN response is highest when homologous virus is used in the assay. VN tests are not standardized in laboratories and are typically used as a research test rather than a diagnostic test. Since the knowledge on PRRSV neutralizing antibody targets and their antigenic variability is incomplete, no clue exists to date to cluster virus isolates into serotypes with respect to antibody-mediated neutralization.

The available literature indicates different PRRSV envelope proteins as targets for neutralizing antibodies, including GP3 (Kim & Yoon, 2008), GP4 (Costers et al., 2010a; Vanhee et al., 2010), GP5 (Gonin et al., 1999; Kim & Yoon, 2008; Kwang et al., 1999; Ostrowski et al., 2002; Pirzadeh & Dea, 1998; Weiland et al., 1999; Yang et al., 2000) and M (Kim & Yoon, 2008; Yang et al., 2000). Based on data of *in vitro* and *in vivo* studies, GP5 is generally seen as the major target for antibody-mediated neutralization of American type PRRSV (Gonin et al., 1999; Ostrowski et al., 2002; Pirzadeh & Dea, 1998). However, it remains to be investigated if this neutralizing epitope of GP5 is also a target for neutralization of European type PRRSV. Nonetheless, the examination of the genomic and predicted amino acid (aa) sequences corresponding to GP5 indicates that the main neutralization epitope is quite conserved among different strains of American type PRRSV. This seems to contradict with the lack of serological cross-reactivity observed between different strains in SN-tests. In EU type PRRSV variants, a region of the GP4 protein was identified as a target for neutralization (Costers et al., 2010; Meulenberg et al., 1997; Vanhee et al., 2010; Weiland et al., 1999). Therefore, precise mapping of neutralizing epitopes is needed

both using monoclonal antibodies and hyper-immune sera obtained from pigs immunized with different strains. In our laboratory, it was demonstrated by pepscan analysis of all envelope glycoproteins that porcine anti-PRRSV antisera (against EU type PRRSV) recognize 21 antigenic regions and that pig differences exist in the recognition pattern (Vanhee et al., 2010; Vanhee et al., 2011). After purifying peptide-specific porcine antibodies, functional analyses were performed. Porcine antibodies that recognize the peptide aa 57-68 of GP4 are strongly neutralizing PRRSV, which was interesting regarding vaccine development (Vanhee et al., 2010). However, presence of a neutralizing antibody-mediated selective pressure on this region of the GP4 protein allows rapid selection of neutralization-resistant variants, which explains the high variability in this region and consequently the lack of cross-neutralization (Costers et al., 2010b; Vanhee et al., 2010). In addition, 2 neutralizing antigenic regions were found in GP2 and 2 others in GP3 (Vanhee et al., 2011). One neutralizing antigenic region in GP3 is highly conserved and neutralizing antibodies against this region were found in most infected pigs. This may be an interesting region for vaccine development. No neutralizing antigenic regions were found in M and GP5 for EU type PRRSV (Vanhee et al., 2011). Differences in cross-protection by polyclonal antibodies between PRRSV strains have recently been demonstrated by Martinez-Lobo and colleagues (2011).

Some studies described that, under certain conditions, PRRSV-specific antibodies may enhance infection (Cancel-Tirado et al., 2004). This phenomenon is called antibody-dependent enhancement of infection (ADEI) and is sometimes observed when neutralizing antisera are diluted beyond the endpoint of neutralization (Yoon et al., 1996). In conclusion, VN antibodies can certainly contribute to resolution of and protection against PRRSV infection, but it appears that also other immune mechanisms (e.g. cell-mediated immunity) are required for an effective defense.

1.2.2.2.2. PASSIVE HUMORAL IMMUNITY

No specific study has evaluated the effect of maternal immunity on piglet susceptibility to PRRSV infection, but indirect inferences suggest that immune sows provide maternal protection to piglets. Houben et al. (1995) discovered a positive link between sow titers and the maternal antibody titers of their litters at 2 weeks of age and the latter were detectable until 4-10 weeks of age. PRRSV maternal antibodies

wane usually at the age of 4 to 5 weeks (Albina et al., 1994). The appearance of PRRSV in weaned pigs has been correlated with loss of maternal antibody and the duration of maternal protection was linked with VN antibody titers (Albina et al. 1994; Chung et al. 1997; Houben et al. 1995). Yoon et al. (1996) demonstrated that infection of porcine alveolar macrophages (PAM) by PRRSV could be enhanced *in vitro* and *in vivo* in the presence of neutralizing antibodies, particularly at low concentrations. These observations suggest that ADEI may play a role in the pathogenesis of the disease.

1.3. PRRSV DIAGNOSIS

A presumptive diagnosis of PRRS is suggested in any herd with reproductive problems in breeding swine and respiratory disease in pigs of any age. Production records in herds with clinically active PRRS usually reveal evidence of increased abortions, early farrowing, stillbirths, pre-weaning mortality, and non-productive sow days. However, the lack of these signs does not indicate that a herd is free of PRRSV infections. Several laboratory methods have been established to detect PRRSV and PRRSV antibodies, including virus isolation (VI) (Mengeling et al., 1999), reverse transcription polymerase chain reaction (RT-PCR) (Sur et al., 1997; Suarez et al., 1996), immunoperoxidase monolayer linked assay (IPMA) (Collins et al., 1992) and enzyme-linked immunosorbent assay (ELISA) (Nodelijk et al., 1996). Virus isolation is most successful using serum, lungs, lymph nodes, and tonsils. Generally, PRRSV is detected in higher amounts and for longer periods in younger, compared to older pigs.

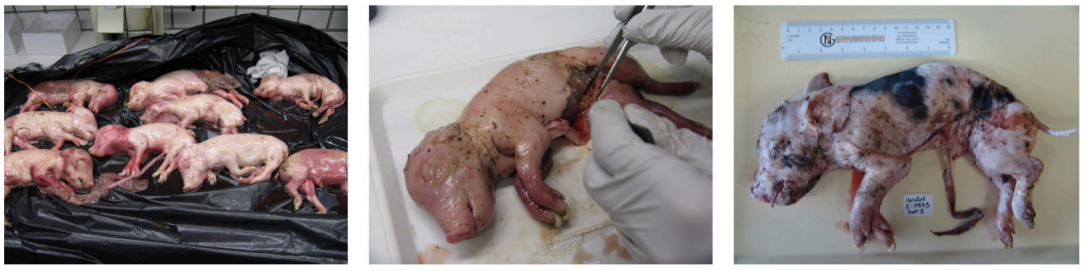


Figure 6. Sample collection of stillborn piglets.

In late-term abortion and early farrowing, similar tissue samples from live-born pigs are preferred, because mummies or stillborn fetuses rarely yield positive VI results due to tissue autolysis. In acute infections, serum and tissues recommended for VI are also the preferred diagnostic samples for PCR. PCR-based assays detect viral nucleic

acids in tissue homogenates, serum, semen, oropharyngeal scrapings, and pulmonary lavage fluids. These assays are highly sensitive and specific (Benson et al., 2002; Horter et al., 2002). However, because of the high degree of sequence variation, the accuracy of RT-PCR might be influenced, especially if mutations are located in the primer or probe binding regions of PCR (Indik et al., 2005). PCR products can be utilized for sequencing, thus expanding the diagnostic utility of this assay. Sequencing is used to show the relatedness of strains over time and within a herd. A phylogenetic tree depicts similarity among genomic sequences like a family lineage. This type of analysis may be used to 1) determine whether the reappearance of PRRS on a farm is due to the re-emergence of a previously existing or a new virus strain; 2) determine whether PRRS outbreaks on farms are due to a single variant or multiple variants of virus; 3) track introduction of (new) virus into a swine herd; 4) monitoring spread of PRRSV strains within and between herds; 5) differentiate vaccine and field viruses (Christopher-Hennings et al., 2002; Roberts, 2001).

Serological diagnosis is still favored by many practitioners because serum is easily collected in quantities for multiple tests and easily stored for future reference. The commercial ELISA (HerdChek® X3 PRRS ELISA, IDEXX Laboratories Inc., Westbrook, Maine) is the "gold standard" for detection of antibodies to PRRSV. The assay is sensitive, specific, standardized, and rapid. The test putatively targets antibodies to the nucleocapsid antigens for both NA and EU type strains of PRRSV. The demonstration of a seroconversion (negative to positive) using acute and convalescent serum samples is the most definitive method to diagnose PRRSV infection serologically. Serology is not a valid approach for diagnosis of PRRSV in previously infected or vaccinated herds, because serological assays do not differentiate among antibodies resulting from the initial infection, reinfection, or vaccination. Detection of antibodies in nursery pigs may be due to the presence of maternal antibodies, which usually persist until pigs reach 3-5 weeks of age (Melnichouk et al., 2005).

Surveillance and monitoring is rarely done in swine populations because of the technical, logistical and economic challenges of bleeding and testing a statistically sufficient number of piglets. A promising 'new' approach for increasing the efficiency and cost-effectiveness of virus surveillance in swine herds and other applications, such as elimination/eradication programs, is oral fluid sampling (Ramirez et al.,

2012). This method is already used for boar stud monitoring due to lower labor time and higher worker safety. ELISA and PCR are typically used in the field to confirm suspicious positive results in herds expected to be negative for PRRSV (Dufresne et al., 2003).

1.4. PRRSV VACCINES

1.4.1. CURRENT COMMERCIAL VACCINES

Two types of PRRSV vaccines are available and widely used in pigs for the control of PRRSV: attenuated or 'modified live virus' (MLV) vaccines, and inactivated or 'killed virus' (KV) vaccines (Christopher-Hennings et al., 1997; Dewey et al., 1999; Kimman et al., 2009; Labarque et al., 2003b; Meng, 2000; Mengeling et al., 1999; Mengeling et al., 2003; Misinzo et al., 2006; Nielsen et al., 2002; Nilubol et al., 2004; van Woensel et al., 1998a; Zuckermann et al., 2007). The former type is generated by *in vitro* cell culture passage of virulent virus until an attenuated phenotype is achieved, while chemically or physically inactivated virulent virus generates the latter type. Inactivated vaccines are administered in combination with an adjuvant, while most attenuated vaccines do not use adjuvants. Both EU type and NA type-based attenuated and inactivated vaccines exist and are used on both continents, since today a strict geographical genotype barrier does no longer exist.

1.4.1.1. ATTENUATED VACCINES

Of both types, attenuated vaccines are clearly the most potent in inducing protection. They are able to significantly reduce viremia, disease occurrence and severity, as well as virus shedding upon challenge with closely related PRRSV strains (Labarque et al., 2003b; Murtaugh et al., 2002; Zuckermann et al., 2007). They also have shown efficacy in reducing mortality and poor growth when vaccinated pigs are exposed to the parental strain from which the vaccine had been prepared (Cano et al., 2007a). The attenuated vaccines are generally effective against genetically related strains (Labarque et al., 2003b; Murtaugh et al., 2002), but are less effective or sometimes ineffective upon challenge with strains that differ genetically (Kimman et al., 2009; Labarque et al., 2004; Murtaugh et al., 2002). Repeated administration of attenuated vaccines within infected populations has also been shown to result in less virus circulation and a reduction of the number of persistently infected pigs (Cano et al.,

2007b). Attenuated live PRRSV vaccines have been successfully employed against the recent emerging HP-PRRSV strains in China and surrounding countries (Leng et al., 2012; Wei et al., 2013). In the study of Li et al. (2013), emergency vaccination with an attenuated live vaccine successfully alleviated the clinical signs of HP-PRRSV infection and reduced the mortality rate. In particular, attenuated vaccines are used to help in the control and elimination of field virus from infected breeding herds (Gillespie & Carroll, 2003). The efficacy of attenuated vaccines is largely subject to the genetic background of the challenge virus and strong to complete protection is only obtained in case the challenge virus is nearly identical to the vaccine virus. The immune response upon vaccination with an attenuated vaccine resembles the same weaknesses as the immunity induced by natural PRRSV infection. They induce non-detectable or low levels of VN antibodies starting from 28 dpi (Lopez & Osorio, 2004; Meier et al., 2003; Zuckermann et al., 2007), which is in line with the PRRSV-intrinsic property of a slow development of low titers of VN antibodies. Remarkably, some studies reported on a strong anamnestic VN antibody response upon challenge, even with a heterologous strain (Osorio et al., 1998), while others reported that the VN antibodies remained unchanged (Zuckermann et al., 2007). VN antibodies induced by attenuated vaccines are often only detected in SN tests using the vaccine virus as antigen, which explains to a certain extent the isolate-specific protective efficacy of these vaccines (Charerntantanakul et al., 2006a; Meier et al., 2003; Okuda et al., 2008; Scortti et al., 2006b; Zuckermann et al., 2007). Similar as in infection-immune animals, the absence of an anamnestic humoral immune response upon challenge or re-vaccination may result from a lack in sufficient replication of the vaccine or virulent virus in vaccination-immune animals (Charerntantanakul et al., 2006a; Scortti et al., 2006b; Zuckermann et al., 2007). In addition to antibody-mediated immunity, attenuated vaccines also induce a cell-mediated immunity, characterized by a gradual development of virus-specific IFN-γ-producing cells and a recall of these cells upon infection. To which extent cell-mediated immune mechanisms contribute to vaccine-induced protection, and which cell types are involved, is not known (Charerntantanakul et al., 2006a; Diaz et al., 2006; Meier et al., 2003; Zuckermann et al., 2007). Nevertheless, attenuated vaccines against PRRSV have several limitations. The current attenuated vaccines behave very similarly to field PRRSV strains in terms of transmission, persistence, transplacental transmission and congenital infection, shedding in semen, and, more importantly, they can revert to

virulence (Bøtner et al., 1997; Christopher-Hennings et al., 1997; Nielsen et al., 2001; Scortti et al., 2006a). Concerning the reversion of the attenuated vaccine strains, an epidemic of the NA genotype PRRSV vaccine occurred in the previously unaffected Danish pig population in 1996 (Bøtner et al., 1997; Nielsen et al., 2001; Nielsen et al., 2002). The circulation of vaccine-derived viruses in Thai swine farms (Amonsin et al., 2009) and the capacity of attenuated vaccines to shape PRRSV evolution by homologous recombination with circulating virus in China (Li et al., 2009; Wenhui et al., 2012) have been reported. Further, vaccine-derived PRRSV strains were isolated from non-symptomatic persistently infected pigs (Key et al., 2001; Zimmerman et al., 2006), and an attenuated vaccine-derived isolate was found to cause disease upon experimental inoculation (Opriessnig et al., 2002). Genetic and phenotypic characterization of isolated field strains suggests that reversion to virulence is not a rare event. Mengeling et al. (1999) used a restriction-site marker specifically present in vaccine virus strain VR2332 to demonstrate the presence of this marker in 24 of 25 field strains isolated after the introduction of the vaccine. More importantly, these putative vaccine-derived strains produced more pronounced pathological changes than did the parental vaccine virus. Wesley et al. (1999) showed that the restriction fragment length polymorphism (RFLP) patterns change when the vaccine virus spreads in a swine population. The frequency of transmission and recombination of attenuated live vaccines should be studied more in depth, as they are worldwide extensively used for the control of the disease. In addition, the potential of spontaneous spreading of either NA or EU genotype attenuated vaccines should be considered in the planning of control or eradication programs using attenuated vaccines (Grosse Beilage et al., 2009). The efficacy of attenuated vaccines is highly dependent on the heterogeneity of the field strain and the safety of these vaccines is not guaranteed. No ready-made answers to any of these problems exist to date.

1.4.1.2. INACTIVATED VACCINES

The outcomes of the use of commercial inactivated vaccines in the field are variable and even less promising than attenuated vaccines. Inactivated PRRSV vaccines have been reported to improve farrowing rate, return to oestrus and piglets weaned per sow in endemically infected populations (Papatsiros et al., 2006), but they generally do not influence viremia, virus replication in tissues and shedding, even when the infectious

PRRSV isolate is nearly homologous to the vaccine strain (Nielsen et al., 1997; Nilubol et al., 2004; Plana-Duran et al., 1997; Scortti et al., 2007; Zuckermann et al., 2007). Inactivated PRRSV vaccines are poorly immunogenic and do not or hardly induce virus-specific antibodies. In several studies, only a moderate anamnestic antibody response is observed upon challenge of vaccinated animals (Meier et al., 2003; Nilubol et al., 2004; Vanhee et al., 2009). Despite the induction of post-challenge PRRSV-neutralizing antibodies (Plana-Duran et al., 1997; Zuckermann et al., 2007) and despite the priming of CD8(high) cells (Piras et al., 2005), they fail to confer protection upon challenge. A non-specific IFN-γ response is induced upon vaccination with a particular inactivated vaccine, though this response is not protective, and is most likely caused by an adjuvant compound rather than by the inactivated virus itself (Piras et al., 2005; Zuckermann et al., 2007). In general, inactivated vaccines are considered less efficacious than attenuated vaccines in prevention of both infection and disease when used in naïve animals (Ostrowski et al., 2002). However, when used in previously infected animals or when used in combination with attenuated vaccines, they may induce more neutralizing antibodies. Several countries only permit the use of inactivated PRRSV vaccines in breeding animals to avoid the use of attenuated PRRSV in gestating sows (the latter may cause reproductive disorders by inappropriate usage). Some inactivated vaccines have been specifically designed for use in sows and gilts to reduce reproductive disorders caused by PRRSV. Inactivated autogenous vaccines can be used in breeding herds in which currently available commercial PRRSV vaccines have failed, particularly to boost immunity from previous natural exposure (M. McCaw, personal communication).

At the moment, several strategies, combining different attenuated and/or inactivated vaccines, for immunizing piglets or gilts and maintaining immunity in sows are applied in the field and have met with variable degrees of success (Martelli et al., 2013; Olanratmanee et al., 2013). PRRSV keeps on circulating in the field, and even farms where strict vaccination procedures are applied may suffer from PRRS-associated problems (Belgian veterinarians in the field, Personal Communications; Thanawongnuwech & Suradhat, 2010). These results may reflect differences in the virus strains circulating in the different farms/regions and/or they may relate to the issue of cross-protection. Taken together, none of the current commercially available vaccines is able to completely prevent respiratory infection, transplacental

transmission, pig-to-pig transmission of the virus, nor maintaining immune protection in sows (Murtaugh et al., 2002). Research to provide a safer and more efficacious product is needed to control the devastating effects of PRRSV.

1.4.2. EXPERIMENTAL VACCINES

As traditional vaccines fail to provide sustainable disease control, novel vaccine development driven by "out of the box" hypotheses, should be encouraged and explored. Several approaches have been used to develop a more effective PRRSV vaccine, including inactivated and attenuated vaccines, DNA vaccines and recombinant DNA vector vaccines (Hu & Zhang, 2013). Although most of these approaches did not result in overwhelming success, they may provide useful lessons.

1.4.2.1. ADAPTABLE INACTIVATED VACCINES

Lopez et al. (2007) demonstrated that neutralizing antibodies have the immunological power to protect pigs upon a homologous challenge. Furthermore, it is well known that maternal immunity is protecting piglets during their first weeks of life. Based on these results, inactivated vaccines that are inducing VN antibodies should be able to give a good protection. However, the commercially available inactivated vaccines do not induce VN antibodies upon vaccination of naïve animals and as a consequence do not protect in these animals. Misinzo et al. (2006) demonstrated the potential of priming the neutralizing antibody response by immunization with a high dose of inactivated PRRSV, leading to certain protection against viremia upon homologous challenge (Misinzo et al., 2006). Since for all viruses VN antibodies are mainly directed against the viral ligands that are involved in binding, internalization and disassembly, these molecules should be preserved during the inactivation process. Delrue et al. (2009) found a method for inactivation of PRRSV without affecting the antigenicity of the envelope proteins, which may lead to a better conservation of neutralizing epitopes (Delrue et al., 2009). Inactivated vaccines produced with this protocol induced VN antibodies in naïve animals and gave a significant protection upon challenge with homologous virus (Vanhee et al., 2009).

1.4.2.2. ADAPTABLE ATTENUATED VACCINES OR VECTOR VACCINES

An improvement of vaccination strategies against PRRSV is required, as current vaccines have limited efficacy. Virus vectored vaccines can represent an advantage to stimulate immune responses against PRRSV. TGEV based vector vaccines expressing different PRRSV antigenic combinations represent a promising candidate to provide protection against PRRSV. Nevertheless, obtained data indicate that heterologous protein expression stability was limited. In swine, pseudorabies virus (PRV) has been used as a vaccine vector for expressing PRRSV immunogens (Qiu et al., 2005). Although a live attenuated vaccine-based PRV recombinant expressing the envelope protein GP5 of PRRSV failed to induce VN antibodies, it conferred partial protection against clinical disease, reduced pathological lesions and the duration of viremia upon PRRSV challenge. Jiang et al. (2008) used replication-defective adenovirus recombinants as vector vaccines to examine the immunogenicity of GP3, GP4, and GP5 in mice. Recently, Wu et al. (2013) used a baculovirus containing a hybrid cytomegalovirus promotor/alphavirus replicon as vector vaccine to examine the immunogenicity of GP5 and M in mice. These strains have, however, not been tested for protective efficacy in pigs. Another vector that has been used to induce immunity to PRRSV is Mycobacterium tuberculosis strain BCG. Following challenge with a PRRSV isolate, M and GP5 immunized pigs showed evidence of partial protection against the PRRSV infection (Bastos et al., 2004).

The results reported to date using viral vectors are not fully satisfactory but cannot be ignored and new vectors must be explored. Deleting the genetic sequences encoding proteins/peptides that (i) are involved in negatively modulating the immune response and that are (ii) associated with virulence will further improve the attenuated PRRSV vaccine. Identification of proteins/peptides that are involved in the induction of a protective immunity (Vanhee et al., 2011; Vanhee et al., 2010) allows introducing their genetic sequences in a vector. At present, a EU project is ongoing, entitled “PoRRSCon, new tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia” with the goal to develop new attenuated and vector vaccines to control PRRS.

1.4.2.3. DNA VACCINES

Thus far, few experimental trials with DNA vaccination have evoked a response strong enough to protect against disease. Kwang et al. (1999) reported both seroconversion and PRRSV-specific lymphocyte proliferation in pigs immunized with PRRSV ORF4, ORF5, ORF6 or ORF7. Nevertheless, only ORF4- and ORF5-immunized pigs developed VN antibodies. Barfoed et al. (2004) cloned all ORFs of a Danish isolate of PRRSV in DNA vaccination vectors. Anamnestic VN antibodies were detected in all pigs, with ORF5-vaccinated pigs showing the highest titers. Rompato et al. (2006) established that an ORF7 vectored vaccine was insufficient in providing protective immunity. Recently, Zhang et al. (2012) illustrated that co-expression of M and IL-18 proteins could significantly improve the potency of DNA vaccination on the activation of vaccine-induced virus-specific cell-mediated immune responses in pigs. In addition, Du et al. (2012) showed that a DNA vaccine expressing GP3, GP5 and interferon α/γ could provide partially protective efficacy. On the other hand, Diaz et al. (2013) has observed that previous immunization with DNA vaccines against ORF5, ORF6, as well as ORF7 but to a lesser degree could result in an exacerbation of the clinical course in terms of fever upon challenge.

1.4.2.4. MARKER VACCINES

For PRRSV eradication, it is desirable to have the possibility to differentiate infected from vaccinated animals (DIVA principle). Marker vaccines together with differentiating ELISAs are interesting tools to start control programs. Of course, one should first identify the most suitable conserved and non-essential epitopes before making a decision on the vaccine. Recent approaches have focused on the non-structural protein 7 (nsp7) of PRRSV that is relatively conserved within the same genotype (EU or NA). Preliminary results indicate that nsp7 can be a good target for setting up a DIVA assay for inactivated vaccines, but further analysis is still needed (M. Garcia Duran, personal communication). In an attempt to develop a marker vaccine, de Lima et al. (2008) developed an attenuated vaccine strain carrying a deletion of an immunodominant B-cell linear epitope in the nsp2 gene. As expected, vaccinated pigs did not develop antibodies to the selected epitope, but the vaccine was not examined for protective capacity. While providing proof of principle of the marker vaccine approach for PRRSV, the nsp2 epitope marker would likely be

suboptimal for detection of exposure of pigs to field strains due to the low degree of conservation and high variability of nsp2, also at the marker site. For that purpose, markers in the conserved M endodomain may be more optimal (de Lima et al., 2008). More recently, Leng et al. (2012) passaged a HP-PRRSV strain on MARC-145 cells with the purpose to use it for the development of an attenuated live vaccine. Sequence analysis of different passages of this HP-PRRSV strain showed that the attenuation resulted in a genetically stable deletion of a continuous 120 aa, in addition to the discontinuous 30-aa deletion in the nsp2 region. This HP-PRRSV vaccine is a good candidate as a marker vaccine against HP-PRRSV.

1.4.2.5. CELL LINES FOR MASS PRODUCTION OF VACCINE VIRUS

Up till now, the immortalized monkey kidney cell line MA-104 and its derivates, MARC-145 and CL2621, are the only continuous cell types shown to support PRRSV infection and are currently employed for virus propagation *in vitro* (Bautista et al., 1993; Kim et al., 1993). Particularly MARC-145 cells are used to grow vaccine virus. Infection of macrophages, the natural host cell of PRRSV, occurs via a few similar but also different receptors compared to infection of MARC-145 cells (Van Breedam et al., 2010). PRRSV first attaches to the macrophage via heparin sulphate (Delputte et al., 2002), then bind to sialoadhesin (Sn) and internalizes (Vanderheijden et al., 2003). CD163 is also involved in infection of macrophages, probably at the stage of virus disassembly (Van Gorp et al., 2008). The entry process of the virus in MARC-145 cells is different from that in macrophages. Most field isolates do not replicate efficiently in MARC-145 cells, or CL 2621 cells, and adaptation is needed to achieve efficient virus replication (Bautista et al., 1993). Sn, which is essential for PRRSV internalization in macrophages, is not detected on MARC-145 cells (Vanderheijden et al., 2003), and sialic acid on PRRSV is essential for infection of primary macrophages, but removal of sialic acid from PRRSV has no effect on infection of MARC-145 cells (Delputte & Nauwynck, 2004). Consequently, virus propagation on MARC-145 cells can lead to mutations of structural proteins that may have an impact on the induction of a protective immunity. In order to overcome several obstacles, a PK15 cell line that is stably transfected with genes encoding the *in vivo* host cell receptors CD163 and Sn has been developed. This cell line is producing large amounts of virus and does not allow significant mutations in the envelope proteins

(Delrue et al., 2010).

In addition, new cell lines have been genetically modified to become permissive to PRRSV, e.g. as immortalized PAM cells expressing the CD163 protein (Lee et al., 2010), immortalized porcine monomyeloid cells expressing the human telomerase reverse transcriptase (Sagong et al., 2012), porcine, feline and baby hamster kidney cells expressing the CD163 protein (Calvert et al., 2007). More recently, a non-genetically modified cell line was tested for PRRSV permissivity (Provost et al., 2012). St-Jude porcine lung cells (SJPL), phenotypically different from MARC-145 cells, are permissive to PRRSV replication and may give us new insight in regards to the viral pathogenesis of PRRSV. In addition, SJPL cells could also serve as a new in vitro model to study viral-bacterial interactions during mixed infections (Provost et al., 2012).

1.4.3. PREREQUISITES FOR FUTURE VACCINES

More than two decades since its emergence, PRRS is still a “mystery swine disease” for most of the vaccinologists and swine veterinarians throughout the world. Numerous reports related to PRRSV vaccine development and field evaluation have yielded disappointing results. The quest for safe and effective PRRSV vaccines goes on, and it is generally agreed to date that the most suitable vaccine should comply with the following criteria (Kimman et al., 2009; Mateu & Diaz, 2008). The vaccine should be **safe**. As indicated above virulent PRRSV can establish persistent infection thanks to its capacity to evade the immune system by mechanisms that are still poorly understood. It is highly unlikely that this feature can be tolerated for any future live PRRSV vaccine. Although PRRSV is not considered as an immunosuppressive virus, any future PRRSV vaccine should not lead to enhanced disease caused by other infections. A better understanding of the interaction of PRRSV and other pathogens therefore is required. Another major goal of any PRRSV vaccination is to induce an immunity that gives a **clinical and virological protection**. A particular challenge to any PRRSV vaccine is to prevent a transplacental transmission of the virus to fetuses. The efficacy of vaccination against reproductive failure can be evaluated by vaccination-challenge studies in pregnant sows. The efficacy against respiratory disease is however more difficult to assess, since other pathogens are usually involved. A very important goal of vaccination is to **diminish the pig-to-pig spread**

of the virus. Further studies to define the role of vaccines in control-eradication programs are therefore needed (Cano et al., 2007a; Cano et al., 2007b). The combination of vaccines, herd closure, biosecurity protocols and air filtration systems are all important to eradicate this virus in certain regions. As previously mentioned, the observed genetic diversity among field isolates is a major obstacle for success of PRRSV vaccines. The effectiveness of a vaccine against heterologous strains will largely depend on the relatedness of the field virus strain to which the vaccinated animals were exposed. New vaccines should always aim to **confer protection against field PRRSV strains that exhibit considerable genetic diversity** (Meng et al., 2000). Alternatively, the composition of the vaccine should be **regularly adapted to actually circulating strains**, as the immune response to PRRSV appears to be, at least partially, strain-dependent to ensure efficacy against the collection of strains circulating at a given moment in time, even between geographically limited areas. Additionally, the ideal vaccine should be **differentiating infected and vaccinated animals**. (DIVA vaccines) (de Lima et al., 2008; Fang et al., 2008).

1.5. REFERENCES

Albina, E., Piriou, L., Hutet, E., Cariolet, R. & L'Hospitalier, R. (1998b). Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Immunol Immunopathol* **61**, 49-66.

Albina, E. (1997). Epidemiology of porcine reproductive and respiratory syndrome (PRRS): an overview. *Vet Microbiol* **55**, 309-316.

Albina, E., Madec, F., Cariolet, R. & Torrison, J. (1994). Immune response and persistence of the porcine reproductive and respiratory syndrome virus in infected pigs and farm units. *Vet Rec* **134**, 567-573.

Allende, R., Laegreid, W. W., Kutish, G. F., Galeota, J. A., Wills, R. W. & Osorio, F. A. (2000). Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. *J Virol* **74**, 10834-10837.

Allende, R., Lewis, T. L., Lu, Z., Rock, D. L., Kutish, G. F., Ali, A., Doster, A. R. & Osorio, F. A. (1999). North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. *J Gen Virol* **80**, 307-315.

Amonsin, A., Kedkovid, R., Puranaveja, S., Wongyanin, P., Suradhat, S. & Thanawongnuwech, R. (2009). Comparative analysis of complete nucleotide sequence of porcine reproductive and respiratory syndrome virus (PRRSV) isolates in Thailand (US and EU genotypes). *Virol J* **6**, 143.

Andreyev, V. G., Wesley, R. D., Mengeling, W. L., Vorwald, A. C. & Lager, K. M. (1997). Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5. *Arch Virol* **142**, 993-1001.

Ansari, I. H., Kwon, B., Osorio, F. A. & Pattnaik, A. K. (2006). Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol* **80**, 3994-4004.

Barfoed, A. M., Blixenkrone-Moller, M., Jensen, M. H., Bøtner, A. & Kamstrup, S. (2004). DNA vaccination of pigs with open reading frame 1-7 of PRRS virus. *Vaccine* **22**, 3628-3641.

Baron, T., Albina, E., Leforban, Y., Madec, F., Guilmoto, H., Duran, J. P. & Vannier, P. (1992). Report on the 1st Outbreaks of the Porcine Reproductive and Respiratory Syndrome (Prrs) in France - Diagnosis and Viral Isolation. *Ann Rech Vet* **23**, 161-166.

Bastos, R. G., Dellagostin, O. A., Barletta, R. G., Doster, A. R., Nelson, E., Zuckermann, F. & Osorio, F. A. (2004). Immune response of pigs inoculated with Mycobacterium bovis BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. *Vaccine* **22**, 467-474.

Batista, L., Pijoan, C., Dee, S., Olin, M., Molitor, T., Joo, H. S., Xiao, Z. & Murtaugh, M. (2004). Virological and immunological responses to porcine reproductive and respiratory syndrome virus in a large population of gilts. *Can J Vet Res* **68**, 267-273.

Baumann, A., Mateu, E., Murtaugh, M. P. & Summerfield, A. (2013). Impact of genotype 1 and 2 of porcine reproductive and respiratory syndrome viruses on interferon-α responses by plasmacytoid dendritic cells. *Vet Res*. **44**, 33.

Bautista, E. M., Suarez, P. & Molitor, T. W. (1999). T cell responses to the structural polypeptides of porcine reproductive and respiratory syndrome virus. *Arch Virol* **144**, 117-134.

Bautista, E. M. & Molitor, T. W. (1997). Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. *Viral Immunol* **10**, 83-94.

Bautista, E. M., Goyal, S. M. & Collins, J. E. (1993). Serologic survey for Lelystad and VR-2332 strains of porcine respiratory and reproductive syndrome (PRRS) virus in US swine herds. *J Vet Diagn Invest* **5**, 612-614.

Benfield, D. A., Nelson, E., Collins, J. E., Harris, L., Goyal, S. M., Robison, D., Christianson, W. T., Morrison, R. B., Gorcyca, D. & Chladek, D. (1992). Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J Vet Diagn Invest* **4**, 127-133.

Benson, J. E., Yaeger, M. J., Christopher-Hennings, J., Lager, K. & Yoon, K. J. (2002). A comparison of virus isolation, immunohistochemistry, fetal serology, and reverse-transcription polymerase chain reaction assay for the identification of porcine reproductive and respiratory syndrome virus transplacental infection in the fetus. *J Vet Diagn Invest* **14**, 8-14.

Bettelli, E., Korn, T., Oukka, M. & Kuchroo, V. K. (2008). Induction and effector functions of T(H)17 cells. *Nature* **453**, 1051-1057.

Beyer, J., Fichtner, D., Schirrmeier, H., Polster, U., Weiland, E. & Wege, H. (2000). Porcine reproductive and respiratory syndrome virus (PRRSV): kinetics of infection in lymphatic organs and lung. *J Vet Med B Infect Dis Vet Public Health* **47**, 9-25.

Bilodeau, R., Archambault, D., Vezina, S. A., Sauvageau, R., Fournier, M. & Dea, S. (1994). Persistence of porcine reproductive and respiratory syndrome virus infection in a swine operation. *Can J Vet Res* **58**, 291-298.

Biron, C. A. (1998). Role of early cytokines, including alpha and beta interferons (IFN-alpha/beta), in innate and adaptive immune responses to viral infections. *Semin Immunol* **10,** 383-390.

Bloemraad, M., de Kluijver, E. P., Petersen, A., Burkhardt, G. E. & Wensvoort, G. (1994). Porcine reproductive and respiratory syndrome: temperature and pH stability of Lelystad virus and its survival in tissue specimens from viraemic pigs. *Vet Microbiol* **42**, 361-371.

Bøtner, A., Strandbygaard, B., Sorensen, K. J., Have, P., Madsen, K. G., Madsen, E. S. & Alexandersen, S. (1997). Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. *Vet Rec* **141**, 497-499.

Brockmeier, S. L., Halbur, P. & Thacker, E. (2002). Porcine Respiratory Disease Complex. In *Polymicrobial Diseases*. Edited by K. A. Brogden & J. M. Guthmiller. Washington (DC): ASM Press.

Burton, D. R. (2002). Antibodies, viruses and vaccines. *Nat Rev Immunol* **2**, 706-13.

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G. &Welch, S. K. (2007). CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *J Virol* **81**, 7371-7379.

Calzada-Nova, G., Schnitzlein, W. M., Husmann, R. J. & Zuckermann, F. A. (2011). North American porcine reproductive and respiratory syndrome viruses inhibit type I interferon production by plasmacytoid dendritic cells. *J Virol* **85**, 2703-2713.

Cancel-Tirado, S. M., Evans, R. B. & Yoon, K. J. (2004). Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. *Vet Immunol Immunopathol* **102**, 249-262.

Cano, J. P., Dee, S. A., Murtaugh, M. P. & Pijoan, C. (2007a). Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. *Vaccine* **25**, 4382-4391.

Cano, J. P., Dee, S. A., Murtaugh, M. P., Trincado, C. A. & Pijoan, C. B. (2007b). Effect of vaccination with a modified-live porcine reproductive and respiratory syndrome virus vaccine on dynamics of homologous viral infection in pigs. *Am J Vet Res* **68**, 565-571.

Cao, J., Jiang, P. & Nauwynck, H. J. (2012). Impaired NK cell-mediated cytotoxicity against PRRSV-infected macrophages in vitro. *22nd International Pig Veterinary Society congress.* July 2012, Jeju, Korea.

Cha, S. H., Choi, E. J., Park, J. H., Yoon, S. R., Song, J. Y., Kwon, J. H., Song, H. J. & Yoon, K. J. (2006). Molecular characterization of recent Korean porcine reproductive and respiratory syndrome (PRRS) viruses and comparison to other Asian PRRS viruses. *Vet Microbiol.* **117**, 248-57.

Chang, C. C., Yoon, K. J., Zimmerman, J. J., Harmon, K. M., Dixon, P. M., Dvorak, C. M. & Murtaugh, M. P. (2002). Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. *J Virol* **76**, 4750-4763.

Charerntantanakul, W., Platt, R., Johnson, W., Roof, M., Vaughn, E. & Roth, J. A. (2006a). Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol* **109**, 99-115.

Chen, N., Cao, Z., Yu, X., Deng, X., Zhao, T., Wang, L., Liu, Q., Li, X., & Tian, K. (2011). Emergence of novel European genotype porcine reproductive and respiratory syndrome virus in mainland China. *J Gen Virol* **92**, 880-892.

Chen, Z. & Plagemann, P. G. (1995). Detection of related positive-strand RNA virus genomes by reverse transcription/polymerase chain reaction using degenerate primers for common replicase sequences. *Virus Res* **39**, 365-375.

Cho, J. G., Dee, S. A. (2006). Porcine reproductive and respiratory syndrome virus. *Theriogenology* **66**, 655-662.

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

Christianson, W. T., Choi, C. S., Collins, J. E., Molitor, T. W., Morrison, R. B. & Joo, H. S. (1993). Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. *Can J Vet Res* **57**, 262-268.

Christianson, W. T., Collins, J. E., Benfield, D. A., Harris, L., Gorcyca, D. E., Chladek, D. W., Morrison, R. B. & Joo, H. S. (1992). Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. *Am J Vet Res* **53**, 485-488.

Christopher-Hennings, J., Faaberg, K. S., Murtaugh, M. P., Nelson, E. A., Roof, M. B., Vaughn, E. M., Yoon, K. J. & Zimmerman, J. (2002). Porcine reproductive and respiratory syndrome (PRRS) diagnostics: Interpretation and limitations. *J Swine Health Prod* **10**, 213-218.

Christopher-Hennings, J., Nelson, E. A., Nelson, J. K. & Benfield, D. A. (1997). Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. *Am J Vet Res* **58**, 40-45.

Christopher-Hennings, J., Nelson, E. A., Hines, R. J., Nelson, J. K., Swenson, S. L., Zimmerman, J. J., Chase, C. L., Yaeger, M. J. & Benfield, D. A. (1995a). Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. *J Vet Diagn Invest* **7**, 456-464.

Christopher-Hennings, J., Nelson, E. A., Nelson, J. K., Hines, R. J., Swenson, S. L., Hill, H. T., Zimmerman, J. J., Katz, J. B., Yaeger, M. J., Chase, C. C. & et al. (1995b). Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. *J Clin Microbiol* **33**, 1730-1734.

Chueh, L. L., Lee, K. H., Wang, F. I., Pang, V. F. & Weng, C. N. (1998). Sequence analysis of the nucleocapsid protein gene of the porcine reproductive and respiratory syndrome virus Taiwan MD-001 strain. *Adv Exp Med Biol* **440**, 795-799.

Chung, W. B., Lin, M. W., Chang, W. F. & Yang, P. C. (1997). Persistence of porcine reproductive and respiratory syndrome virus in intensive farrow-to-finish pig herds. *Can J Vet Res* **61**, 292–298.

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., Goyal, S. M., McCullough, S., Morrison, R. B., Joo, H. S. & et al. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest* **4**, 117-126.

Conzelmann, K. K., Visser, N., Van Woensel, P. & Thiel, H. J. (1993). Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. *Virology* **193**, 329-339.

Costers, S., Lefebvre, D. J., Van Doorsselaere, J., Vanhee, M., Delputte, P. L. & Nauwynck, H. J. (2010a). GP4 of porcine reproductive and respiratory syndrome virus

contains a neutralizing epitope that is susceptible to immunoselection in vitro. *Arch Virol* **155**, 371-378.

Costers, S., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Geldhof, M. & Nauwynck, H. J. (2010b). GP4-specific neutralizing antibodies might be a driving force in PRRSV evolution. *Virus Res* **154**, 104-113.

Costers, S., Lefebvre, D. J., Goddeeris, B., Delputte, P. L. & Nauwynck, H. J. (2009). Functional impairment of PRRSV-specific peripheral CD3+CD8high cells. *Vet Res* **40**, 46.

Darwich, L., Díaz, I. & Mateu, E. (2010). Certainties, doubts and hypotheses in porcine reproductive and respiratory syndrome virus immunobiology. *Virus Res* **154**, 123-132.

Dea, S., Bilodeau, R., Athanassious, R., Sauvageau, R. A. & Martineau, G. P. (1992). Isolation of the Porcine Reproductive and Respiratory Syndrome Virus in Quebec. *Canadian Veterinary Journal-Revue Veterinaire Canadienne* **33**, 552-553.

Dee, S., Otake, S. & Deen, J. (2010). Use of a production region model to assess the efficacy of various air filtration systems for preventing airborne transmission of porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae: results from a 2-year study. *Virus Res* **154**, 177-184.

de Lima, M., Kwon, B., Ansari, I. H., Pattnaik, A. K., Flores, E. F. & Osorio, F. A. (2008). Development of a porcine reproductive and respiratory syndrome virus differentiable (DIVA) strain through deletion of specific immunodominant epitopes. *Vaccine* **26**, 3594-3600.

Delputte, P. L. & Nauwynck, H. J. (2004). Porcine arterivirus infection of alveolar macrophages is mediated by sialic acid on the virus. *J Virol* **78**, 8094-8101.

Delputte, P. L., Meerts, P., Costers, S. & Nauwynck, H. J. (2004). Effect of virus-specific antibodies on attachment, internalization and infection of porcine reproductive and respiratory syndrome virus in primary macrophages. *Vet Immunol Immunopathol* **102**, 179-188.

Delputte, P. L., Vanderheijden, N., Nauwynck, H. J. & Pensaert, M. B. (2002). Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J Virol* **76**, 4312-4320.

Delrue, I., Van Gorp, H., Van Doorsselaere, J., Delputte, P. L. & Nauwynck, H. J. (2010). Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol* **10**, 48.

Delrue, I., Delputte, P. L. & Nauwynck, H. J. (2009). Assessing the functionality of viral entry-associated domains of porcine reproductive and respiratory syndrome virus during inactivation procedures, a potential tool to optimize inactivated vaccines. *Vet Res* **40**, 62.

Dewey, C., Charbonneau, G., Carman, S., Hamel, A., Nayar, G., Friendship, R., Eernisse, K. & Swenson, S. (2000). Lelystad-like strain of porcine reproductive and respiratory syndrome virus (PRRSV) identified in Canadian swine. *Can Vet J* **41**, 493-4.

Dewey, C. E., Wilson, S., Buck, P. & Leyenaar, J. K., (1999). The reproductive performance of sows after PRRS vaccination depends on stage of gestation. *Prev Vet Med* **40**, 233-241.

Diaz, I., Ganges, L., Galindo-Cardiel, I., Tarradas, J., Alvarez, B., Lorca-Oró, C., Pujols, J., Gimeno, M., Darwich, L., Domingo, M., Domínguez, J. & Mateu, E. (2013). Immunization with DNA vaccines containing porcine reproductive and respiratory syndrome virus open reading frames 5, 6, and 7 may be related to the exacerbation of clinical disease after an experimental challenge. *Viral Immunol* **26**, 93-101.

Diaz, I., Pujols, J., Ganges, L., Gimeno, M., Darwich, L., Domingo, M. & Mateu, E. (2009). In silico prediction and ex vivo evaluation of potential T-cell epitopes in glycoproteins 4 and 5 and nucleocapsid protein of genotype-I (European) of porcine reproductive and respiratory syndrome virus. *Vaccine* **27**, 5603-5611.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. (2006). Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. *Virology* **351**, 249-259.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. (2005). Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. *J Gen Virol* **86**, 1943-1951.

Dokland, T. (2010). The structural biology of PRRSV. *Virus Res* **154**, 86-97.

Done, S. H. & Paton, D. J. (1995). Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. *Vet Rec* **136**, 32-35.

Du, Y., Qi, J., Lu, Y., Wu, J., Yoo, D., Liu, X., Zhang, X., Li, J., Sun, W., Cong, X., Shi, J. & Wang, J. (2012). Evaluation of a DNA vaccine candidate co-expressing GP3 and GP5 of porcine reproductive and respiratory syndrome virus (PRRSV) with interferon α/γ in immediate and long-lasting protection against HP-PRRSV challenge. *Virus Genes* **45**, 474-487.

Duan, X., Nauwynck, H. J. & Pensaert, M. B. (1997a). Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). *Arch Virol* **142**, 2483-2497.

Duan, X., Nauwynck, H. J. & Pensaert, M. B. (1997b). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Microbiol* **56**, 9-19.

Dufresne, L., Polson, D. D., Holck, J. T. & Roberts, J. (2003). Serological monitoring in negative and low prevalence populations. In The PRRS Compendium (2nd edition). J Zimmerman, K-J Yoon, eds. Des Moines, Iowa: National Pork Board, pp. 87-101.

Dwivedi, V., Manickam, C., Binjawadagi, B., Linhares, D., Murtaugh, M. P. & Renukaradhya, G. J. (2012). Evaluation of immune responses to porcine reproductive and respiratory syndrome virus in pigs during early stage of infection under farm conditions. *Virol J* **9**:45.

Epperson, B. & Holler, L. (1997). An abortion stormand sow mortality syndrome. *Proc Am Assoc Swine Pract* **28**, 479-483.

Faaberg, K. S., Hocker, J. D., Erdman, M. M., Harris, D. L., Nelson, E. A., Torremorell, M. & Plagemann, P. G. (2006). Neutralizing antibody responses of pigs infected with natural GP5 N-glycan mutants of porcine reproductive and respiratory syndrome virus. *Viral Immunol* **19**, 294-304.

Fang, Y., Christopher-Hennings, J., Brown, E., Liu, H., Chen, Z., Lawson, S. R., Breen, R., Clement, T., Gao, X., Bao, J., Knudsen, D., Daly, R. & Nelson, E. (2008). Development of genetic markers in the non-structural protein 2 region of a US type 1 porcine reproductive and respiratory syndrome virus: implications for future recombinant marker vaccine development. *J Gen Virol* **89**, 3086-3096.

Fitzgerald-Bocarsly, P. & Feng, D. (2007). The role of type I interferon production by dendritic cells in host defense. *Biochemie* **89**, 843-855.

Flores-Mendoza, L., Silva-Campa, E., Resendiz, M., Osorio, F. A. & Hernandez, J. (2008). Porcine reproductive and respiratory syndrome virus infects mature porcine dendritic cells and up-regulates interleukin-10 production. *Clin Vaccine Immunol* **15**, 720-725.

Forsberg, R. (2005). Divergence time of porcine reproductive and respiratory syndrome virus subtypes. *Mol Biol Evol* **22**, 2131-2134.

Gao, Z. Q., Guo, X. & Yang, H. C. (2004). Genomic characterization of two Chinese isolates of porcine respiratory and reproductive syndrome virus. *Arch Virol* **149**, 1341-1351.

Gillespie, T. G. & Carroll, A. L. (2003). Methods of control and elimination of porcine reproductive and respiratory syndrome virus using modified live vaccine in a two-site production system. *J Swine health Prod* **11**, 291-295.

Gimeno, M., Darwich, L., Diaz, I., de la Torre, E., Pujols, J., Martin, M., Inumaru, S., Cano, E., Domingo, M., Montoya, M. & Mateu, E. (2011). Cytokine profiles and phenotype regulation of antigen presenting cells by genotype-I porcine reproductive and respiratory syndrome virus isolates. *Vet Res* **42**, 9.

Goldberg, T. L., Lowe, J. F., Milburn, S. M. & Firkins, L. D. (2003). Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection. *Virology* **317**, 197-207.

Gonin, P., Pirzadeh, B., Gagnon, C. A. & Dea, S. (1999). Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. *J Vet Diagn Invest* **11**, 20-26.

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J. & Snijder, E. J. (2006). Nidovirales: evolving the largest RNA virus genome. *Virus Res* **117**, 17-37.

Gravell, M., London, W. T., Leon, M. E., Palmer, A. E. & Hamilton, R. S. (1986). Differences among isolates of simian hemorrhagic fever (SHF) virus. *Proc Soc Exp Biol Med* **181**, 112-119.

Grosse Beilage, E., Nathues, H., Meemken, D., Harder, T. C., Doherr, M. G., Grotha, I. & Greiser-Wilke, I. (2009). Frequency of PRRS live vaccine virus (European and North American genotype) in vaccinated and non-vaccinated pigs submitted for respiratory tract diagnostics in North-Western Germany. *Prev Vet Med* **92**, 31-37.

Halbur, P. G. & Bush, E. (1997). Update on abortion storms and sow mortality. *Swine Health and Production* **5**, 73.

Halbur, P. G., Paul, P. S., Frey, M. L., Landgraf, J., Eernisse, K., Meng, X. J., Andrews, J. J., Lum, M. A. & Rathje, J. A. (1996a). Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet Pathol* **33**, 159-170.

Halbur, P. G., Paul, P. S., Meng, X. J., Lum, M. A., Andrews, J. J. & Rathje, J. A. (1996b). Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. *J Vet Diagn Invest* **8**, 11-20.

Halbur, P. G., Miller, L. D., Paul, P. S., Meng, X. J., Huffman, E. L. & Andrews, J. J. (1995). Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrumdeprived pigs. *Vet Pathol* **32**, 200-204.

Hanada, K., Suzuki, Y., Nakane, T., Hirose, O. & Gojobori, T. (2005). The origin and evolution of porcine reproductive and respiratory syndrome viruses. *Mol Biol Evol* **22**, 1024-1031.

Hill, H. T. (1990). Overview and history of mystery swine disease (Swine Infertility Respiratory Syndrome). In Proceedings Mystery Swine disease Committee Meeting, pp. 29-31. Denver, Colorado, USA.

Hopper, S. A., White, M. E. C. & Twiddy, N. (1992). An Outbreak of Blue-Eared Pig-Disease (Porcine Reproductive and Respiratory Syndrome) in 4 Pig Herds in Great-Britain. *Veterinary Record* **131**, 140-144.

Horter, D. C., Pogranichniy, R. M., Chang, C. C., Evans, R. B., Yoon, K. J. & Zimmerman, J. J. (2002). Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. *Vet Microbiol* **86**, 213-228.

Houben, S., van Reeth, K. & Pensaert, M. B. (1995). Pattern of infection with the porcine reproductive and respiratory syndrome virus on swine farms in Belgium. *Zentralbl Veterinarmed B* **42**, 209-215.

Hu, J. & Zhang, C. (2013). Porcine reproductive and respiratory syndrome virus vaccines: current status and strategies to a universal vaccine. *Transbound Emerg Dis* doi: 10.1111/tbed.12016.

Indik, S., Schmoll, F., Sipos, W., & Klein, D. (2005). Genetic variability of PRRS virus in Austria: consequences for molecular diagnostics and viral quantification. *Vet Microbiol* **107**, 171-8.

Indik, S., Valicek, L., Klein, D. & Klanova, J. (2000). Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **81**, 2497-2502.

Janeway, C. A., Travers, P., Walport, M. & Schlomchik, M. (2005). Immunobiology, the immune system in health and disease; 6th edition. New York, USA: *Garland Science Publishing*

Jiang, W., Jiang, P., Wang, X., Li, Y., Du, Y. & Wang, X. (2008). Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. *Virus Res* **136**, 50-57.

Jiang, P., Chen, P. Y., Dong, Y. Y., Cai, J. L., Cai, B. X. & Jiang, Z. H. (2000). Isolation and genome characterization of porcine reproductive and respiratory syndrome virus in P. R. China. *J Vet Diagn Invest* **12**, 156-158.

Johnson, C. R., Griggs, T. F., Gnanandarajah, J., Murtaugh, M. P. (2011). Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. *J Gen Virol* **92**, 1107-1116.

Joo, H. S., Park, B. K., Dee, S. A. & Pijoan, C. (1997). Indirect fluorescent IgM antibody response of pigs infected with porcine reproductive and respiratory syndrome syndrome virus. *Vet Microbiol* **55**, 303-307.

Jung, K., Renukaradhya, G. J., Alekseev, K. P., Fang, Y., Tang, Y. & Saif, L. J. (2009). Porcine reproductive and respiratory syndrome virus modifies innate immunity and alters disease outcome in pigs subsequently infected with porcine respiratory coronavirus: implications for respiratory viral co-infections. *J Gen Virol* **90**, 2713-2723.

Kapur, V., Elam, M. R., Pawlovich, T. M. & Murtaugh, M. P. (1996). Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. *J Gen Virol* **77**, 1271-1276.

Karniychuk, U. U., Saha, D., Geldhof, M., Vanhee, M., Cornillie, P., Van den Broeck, W. & Nauwynck, H. J. (2011). Porcine reproductive and respiratory syndrome virus (PRRSV) causes apoptosis during its replication in fetal implantation sites *Microb Pathog* **51**, 194-202.

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A. & Nauwynck, H. J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. *BMC Vet Res* **6**, 30.

Karniychuk, U. U. & Nauwynck, H. J. (2009). Quantitative changes of sialoadhesin and CD163 positive macrophages in the implantation sites and organs of porcine embryos/fetuses during gestation. *Placenta* **30**, 497-500.

Keffaber, K. K. (1989). Reproductive failure of unknown ethiology. *American Association of Swine Practitioners Newsletter* **1**, 1-10.

Key, K. F., Haqshenas, G., Guenette, D. K., Swenson, S. L., Toth, T. E. & Meng, X. J. (2001). Genetic variation and phylogenetic analyses of the ORF5 gene of acute porcine reproductive and respiratory syndrome virus isolates. *Vet Microbiol* **83**, 249-263.

Kim, H. S., Kwang, J., Yoon, I. J., Joo, H. S. & Frey, M. L. (1993). Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch Virol* **133**, 477-483.

Kim, W. I. & Yoon, K. J. (2008). Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. *Virus Genes* **37**, 380-391.

Kimman, T. G., Cornelissen, L. A., Moormann, R. J., Rebel, J. M. & Stockhofe-Zurwieden, N. (2009). Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. *Vaccine* **27**, 3704-3718.

Kwang, J., Yang, S., Osorio, F. A., Christian, S., Wheeler, J. G., Lager, K. M., Low, S., Chang, L., Doster, A. R., White, A. & Wu, C. C. (1999). Characterization of antibody response to porcine reproductive and respiratory syndrome virus ORF5 product following infection and evaluation of its diagnostic use in pigs. *J Vet Diagn Invest* **11**, 391-395.

Labarque, G., Reeth, K. V., Nauwynck, H., Drexler, C., Van Gucht, S. & Pensaert, M. (2004). Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. *Vaccine* **22**, 4183-4190.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H. & Pensaert, M. (2003b). Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. *Vet Microbiol* **95**, 187-197.

Labarque, G. G., Nauwynck, H. J., Van Reeth, K. & Pensaert, M. B. (2000). Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. *J Gen Virol* **81**, 1327-1334.

Lager, K. M., Mengeling, W. L. & Brockmeier, S. L. (1999). Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. *Am J Vet Res* **60**, 1022-1027.

Lager, K. M. & Mengeling, W. L. (1995). Pathogenesis of in utero infection in porcine fetuses with porcine reproductive and respiratory syndrome virus. *Can J Vet Res* **59**, 187-192.

Lawson, S. R., Rossow, K. D., Collins, J. E., Benfield, D. A. & Rowland, R. R. (1997). Porcine reproductive and respiratory syndrome virus infection of gnotobiotic pigs: sites of virus replication and co-localization with MAC-387 staining at 21 days post-infection. *Virus Res* **51**, 105-113.

Lee, Y. J., Park, C. K., Nam, E., Kim, S. H., Lee, O. S., du Lee, S. & Lee, C. (2010). Generation of a porcine alveolar macrophage cell line for the growth of porcine reproductive and respiratory syndrome virus. *J Virol Methods* **163**, 410-415.

Leng, X., Li, Z., Xia, M., He, Y. & Wu, H. (2012). Evaluation of the efficacy of an attenuated live vaccine against highly pathogenic porcine reproductive and respiratory syndrome virus in young pigs. *Clin Vaccine Immunol* **19**, 1199-1206.

Li, X., Qiu, L., Yang, Z., Dang, R. & Wang, X. (2013). Emergency vaccination alleviates highly pathogenic porcine reproductive and respiratory syndrome virus infection after contact exposure. *BMC Vet Res* **9**, 26.

Li, J., Jiang, P., Li, Y., Wang, X., Cao, J. & Zeshan, B. (2009). HSP70 fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus enhanced the immune responses and protective efficacy against virulent PRRSV challenge in pigs. *Vaccine* **27**, 825-832.

Linhares, D. C., Cano, J. P., Wetzell, T., Nerem, J., Torremorell, M. & Dee, S. A. (2012). Effect of modified-live porcine reproductive and respiratory syndrome virus (PRRSv) vaccine on the shedding of wild-type virus from an infected population of growing pigs. *Vaccine* **30**, 407-413.

Lodoen, M. B. & Lanier, L. L. (2006). Natural killer cells as an initial defense against pathogens. *Curr Opin Immunol* **18**, 391-398.

Loemba, H. D., Mounir, S., Mardassi, H., Archambault, D. & Dea, S. (1996). Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. *Arch Virol* **141**, 751-761.

Lopez, O. J., Oliveira, M. F., Garcia, E. A., Kwon, B. J., Doster, A. & Osorio, F. A. (2007). Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. *Clin Vaccine Immunol* **14**, 269-275.

Lopez, O. J. & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol* **102**, 155-163.

Lopez-Fuertes, L., Campos, E., Domenech, N., Ezquerra, A., Castro, J. M., Dominguez, J. & Alonso, F. (2000). Porcine reproductive and respiratory syndrome (PRRS) virus down-modulates TNF-alpha production in infected macrophages. *Virus Res* **69**, 41-46.

Madsen, K. G., Hansen, C. M., Madsen, E. S., Strandbygaard, B., Bøtner, A. & Sørensen, K. J. (1998). Sequence analysis of porcine reproductive and respiratory

syndrome virus of the American type collected from Danish swine herds. *Arch Virol* **143**, 1683-1700.

Mardassi, H., Athanassious, R., Mounir, S. & Dea, S. (1994). Porcine reproductive and respiratory syndrome virus: morphological, biochemical and serological characteristics of Quebec isolates associated with acute and chronic outbreaks of porcine reproductive and respiratory syndrome. *Can J Vet Res* **58**, 55-64.

Martelli, P., Ardigò, P., Ferrari, L., Morganti, M., De Angelis, E., Bonilauri, P., Luppi, A., Guazzetti, S., Caleffi, A. & Borghetti, P. (2013). Concurrent vaccinations against PCV2 and PRRSV: study on the specific immunity and clinical protection in naturally infected pigs. *Vet Microbiol* **162**, 558-571.

Martinez-Lobo, F. J., Diez-Fuertes, F., Simarro, I., Castro, J. M., & Prieto, C. (2011). Porcine reproductive and respiratory syndrome virus isolates differ in their susceptibility to neutralization. *Vaccine* **29**, 6928-6940.

Mateu, E. & Diaz, I. (2008). The challenge of PRRS immunology. *Vet J* **177**, 345-351.

Meier, W. A., Galeota, J., Osorio, F. A., Husmann, R. J., Schnitzlein, W. M. & Zuckermann, F. A. (2003). Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. *Virology* **309**, 18-31.

Melnichouk, O., Dewey, C. E., Friendship, R. M. & Haydon, D. T. (2005). Sero-epidemiological study of porcine reproductive and respiratory syndrome (PRRS) virus infection patterns by age in nursery pigs on seven commercial farms in Ontario, Canada. *Prev Vet Med* **62**, 57-62.

Meng, X. J. (2000). Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. *Vet Microbiol* **12**, 309-329.

Meng, X. J., Paul, P. S., Halbur, P. G. & Morozov, I. (1995). Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **76**, 3181-3188.

Meng, X. J., Paul, P. S. & Halbur, P. G. (1994). Molecular cloning and nucleotide sequencing of the 3'-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. *J Gen Virol* **75**, 1795-1801.

Mengeling, W. L., Lager, K. M., Vorwald, A. C. & Koehler, K. J. (2003). Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **93**, 13-24.

Mengeling, W. L., Vorwald, A. C., Lager, K. M., Clouser, D.F. & Wesley, R. D. (1999). Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **60**, 334-340.

Mengeling, W. L., Lager, K. M., Wesley, R. D., Clouser, D. F., Vorwald, A. C. & Roof, M. B. (1999). Diagnostic implications of concurrent inoculation with attenuated and virulent strains of porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **60**, 119-122.

Mengeling, W. L., Lager, K. M. & Vorwald, A. C. (1998). Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of "atypical" PRRS. *Am J Vet Res* **59**, 1540-1544.

Mengeling, W. L., Lager, K. M. & Vorwald, A. C. (1994). Temporal characterization of transplacental infection of porcine fetuses with porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **55**, 1391-1398.

Meulenberg, J. J., van Nieuwstadt, A. P., van Essen-Zandbergen, A. & Langeveld, J. P. (1997). Posttranslational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. *J Virol* **71**, 6061-6067.

Meulenberg, J. J. & Petersen-den Besten, A. (1996). Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP2 encoded by ORF2 is incorporated in virus particles. *Virology* **225**, 44-51.

Meulenberg, J. J., Petersen-den Besten, A., De Kluyver, E. P., Moormann, R. J., Schaaper, W. M. & Wensvoort, G. (1995). Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. *Virology* **206**, 155-163.

Meulenberg, J. J., Hulst, M. M., de Meijer, E. J., Moonen, P. L., den Besten, A., de Kluyver, E. P., Wensvoort, G. & Moormann, R. J. (1993). Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. *Virology* **192**, 62-72.

Misinzo, G., Delputte, P. L., Meerts, P., Drexler, C. & Nauwynck, H. J. (2006). Efficacy of an inactivated PRRSV vaccine: induction of virus-neutralizing antibodies and partial virological protection upon challenge. *Adv Exp Med Biol* **581**, 449-454.

Morozov, I., Meng, X. J. & Paul, P. S. (1995). Sequence analysis of open reading frames (ORFs) 2 to 4 of a U.S. isolate of porcine reproductive and respiratory syndrome virus. *Arch Virol* **140**, 1313-1319.

Mulupuri, P., Zimmerman, J. J., Hermann, J., Johnson, C. R., Cano, J. P., Yu, W., Dee, S. A. & Murtaugh, M. P. (2008). Antigen-specific B-cell responses to porcine reproductive and respiratory syndrome virus infection. *J Virol* **82**, 358-370.

Murtaugh, M. P., Stadejek, T., Abrahante, J. E., Lam, T. T. & Leung, F. C. (2010). The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. *Virus Res* **154**, 18-30.

Murtaugh, M. P., Xiao, Z. & Zuckermann, F. (2002). Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol* **15**, 533-547.

Nelsen, C. J., Murtaugh, M. P. & Faaberg, K. S. (1999). Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol* **73**, 270-280.

Nelson, E. A., Christopher-Hennings, J. & Benfield, D. A. (1994). Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. *J Vet Diagn Invest* **6**, 410-415.

Neumann, E. J., Kliebenstein, J. B., Johnson, C. D., Mabry, J. W., Bush, E. J., Seitzinger, A. H., Green, A. L. & Zimmerman, J. J. (2005). Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. *J Am Vet Med Assoc* **227**, 385-392.

Newman, K. C. & Riley, E. M. (2007). Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat Rev Immunol* **7**, 279-291.

Nielsen, H. S., Oleksiewicz, M. B., Forsberg, R., Stadejek, T., Bøtner, A. & Storgaard, T. (2001). Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J Gen Virol* **82**, 1263-1272.

Nielsen, J., Bøtner, A., Bille-Hansen, V., Oleksiewicz, M. B. & Storgaard, T. (2002). Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus. *Vet Microbiol* **84**, 1-13.

Nielsen, T. L., Nielsen, J., Have, P., Baekbo, P., Hoff-Jorgensen, R. & Bøtner, A. (1997). Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **54**, 101-112.

Nilubol, D., Platt, K. B., Halbur, P. G., Torremorell, M. & Harris, D. L. (2004). The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. *Vet Microbiol* **102**, 11-18.

Nodelijk, G., Nielen, M., De Jong, M. C. & Verheijden, J. H. (2003). A review of porcine reproductive and respiratory syndrome virus in Dutch breeding herds: population dynamics and clinical relevance. *Prev Vet Med* **60**, 37-52.

Nodelijk, G., Wensvoort, G., Kroese, B., van Leengoed, L., Colijn, E. & Verheijden, J. (1996). Comparison of a commercial ELISA and an immunoperoxidase monolayer assay to detect antibodies directed against porcine respiratory and reproductive syndrome virus. *Vet Microbiol* **49**, 285-295.

Normile, D. (2007). China, Vietnam grapple with 'rapidly evolving' pig virus. *Science* **317**, 1017.

Okuda, Y., Kuroda, M., Ono, M., Chikata, S. & Shibata, I. (2008). Efficacy of vaccination with porcine reproductive and respiratory syndrome virus following challenges with field isolates in Japan. *J Vet Med Sci* **70**, 1017-1025.

Olanratmanee, E. O., Nuntawan Na Ayudhya, S., Thanawongnuwech, R., Kunavongkrit, A. & Tummaruk, P. (2013). Reproductive parameters following a PRRS outbreak where a whole-herd PRRS MLV vaccination strategy was instituted post-outbreak. *Trop Anim Health Prod* **45**, 1099-1106.

Oleksiewicz, M. B., Bøtner, A., Toft, P., Normann, P. & Storgaard, T. (2001). Epitope mapping porcine reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the replicase polyprotein contains a cluster of B-cell epitopes. *J Virol* **75**, 3277-3290.

Onyekaba, C. O., Harty, J. T., Even, C., Hu, B. G. & Plagemann, P. G. (1989). Persistent infection of mice by lactate dehydrogenase-elevating virus: effects of immunosuppression on virus replication and antiviral immune responses. *Virus Res* **14**, 297-315.

Opriessnig, T., Halbur, P. G., Yoon, K. J., Pogranichniy, R. M., Harmon, K. M., Evans, R., Key, K. F., Pallares, F. J., Thomas, P. & Meng, X. J. (2002). Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. *J Virol* **76**, 11837-11844.

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F. & Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology* **302**, 9-20.

Osorio, F. A., Zuckermann, F., Wills, R., Meier, W., Christian, S., Galeota, S. &Doster, A. (1998). PRRSV: comparison of commercial vaccines in their ability to induce protection against current PRRSV strains of high virulence. In: *Leman, D.A. (Ed.) Swine Conf.* **25**, 176–182.

Ostrowski, M., Galeota, J. A., Jar, A. M., Platt, K. B., Osorio, F. A. & Lopez, O. J. (2002). Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol* **76**, 4241-4250.

Otake, S., Dee, S., Corzo, C., Oliveira, S. & Deen, J. (2010). Long-distance airborne transport of infectious PRRSV and Mycoplasma hyopneumoniae from a swine population infected with multiple viral variants. *Vet Microbiol* **145**, 198-208.

Otake, S., Dee, S. A., Rossow, K. D., Deen, J., Joo, H. S., Molitor, T. W. & Pijoan, C. (2002a). Mechanical transmission of porcine reproductive and respiratory syndrome virus by fomites (boots and overalls). *J Swine Health Prod* **10**, 59-65.

Otake, S., Dee, S. A., Rossow, K. D., Moon, R. & Pijoan, C. (2002b). Transmission of porcine reproductive and respiratory syndrome virus by mosquitoes, *Aedes vexans* (Meigen). *Can J Vet Res* **66**, 191-195.

Papatsiros, V. G., Alexopoulos, C., Kritas, S. K., Koptopoulos, G., Nauwynck, H. J., Pensaert, M. B. & Kyriakis, S. C. (2006). Long-term administration of a commercial porcine reproductive and respiratory syndrome virus (PRRSV)-inactivated vaccine in PRRSV-endemically infected sows. *J Vet Med* **53**, 266-272.

Paton, D. J. & Drew, T. W. (1995). Serological monitoring of PRRS transmission: a case study. *Vet Rec* **136**, 297-298.

Pejsak, Z., Stadejek, T. & Markowska-Daniel, I. (1997). Clinical signs and economic losses caused by porcine reproductive and respiratory syndrome virus in a large breeding farm. *Vet Microbiol* **55**, 317-322.

Pintaric, M., Gerner, W. & Saalmuller, A. (2008). Synergistic effects of IL-2, IL-12 and IL-18 on cytolytic activity, perforin expression and IFN-gamma production of porcine natural killer cells. *Vet Immunol Immunopathol* **121**, 68-82.

Piras, F., Bollard, S., Laval, F., Joisel, F., Reynaud, G., Charreyre, C., Andreoni, C. & Juillard, V. (2005). Porcine reproductive and respiratory syndrome (PRRS) virus-specific interferon-gamma(+) T-cell responses after PRRS virus infection or vaccination with an inactivated PRRS vaccine. *Viral Immunol* **18**, 381-389.

Pirzadeh, B. & Dea, S. (1998). Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **79**, 989-999.

Plana, J., Vayreda, M., Vilarrasa, J., Bastons, M., Rosell, R., Martinez, M., Gabriel, A. S., Pujols, J., Badiola, J. L., Ramos, J. A. & Domingo, M. (1992). Porcine Epidemic Abortion and Respiratory Syndrome (Mystery Swine Disease) - Isolation in Spain of the Causative Agent and Experimental Reproduction of the Disease. *Vet Microbiol* **33**, 203-211.

Plana-Duran, J., Bastons, M., Urniza, A., Vayreda, M., Vila, X. & Mane, H. (1997). Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **55**, 361-370.

Prieto, C., Vázquez, A., Núñez, J. I., Alvarez, E., Simarro, I. & Castro, J. M. (2009). Influence of time on the genetic heterogeneity of Spanish porcine reproductive and respiratory syndrome virus isolates. *Vet J* **180**, 363-370.

Prieto, C., Alvarez, E., Martinez-Lobo, F. J., Simarro, I. & Castro, J. M. (2008). Similarity of European porcine reproductive and respiratory syndrome virus strains to vaccine strain is not necessarily predictive of the degree of protective immunity conferred. *Vet J* **175**, 356-363.

Prieto, C. & Castro, J. M. (2005). Porcine reproductive and respiratory syndrome virus infection in the boar: a review. *Theriogenology* **63**, 1-16.

Prieto, C., Suarez, P., Simarro, I., Garcia, C., Martin-Rillo, S. & Castro, J. M. (1997). Insemination of susceptible and preimmunized gilts with boar semen containing porcine reproductive and respiratory syndrome virus. *Theriogenology* **47**, 647-654.

Provost, C., Jia, J. J., Music, N., Lévesque, C., Lebel, M. È., del Castillo, J. R., Jacques, M. & Gagnon, C. A. (2012). Identification of a new cell line permissive to porcine reproductive and respiratory syndrome virus infection and replication which is phenotypically distinct from MARC-145 cell line. *Virol J* **9**, 267.

Qiu, H. J., Tian, Z. J., Tong, G. Z., Zhou, Y. J., Ni, J. Q. & Luo J. Z. et al. (2005). Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. *Vet Immunol Immunopathol* **106**, 309-319.

Ramirez, A., Wang, C., Prickett, J. R., Pogranichniy, R., Yoon, K. J., Main, R., Johnson, J. K., Rademacher, C., Hoogland, M., Hoffmann, P., Kurtz, A., Kurtz, E. & Zimmerman, J. (2012). Efficient surveillance of pig populations using oral fluids. *Prev Vet Med* **104**, 292-300.

Renukaradhya, G. J., Alekseev, K., Jung, K., Fang, Y. & Saif, L. J. (2010). Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. *Viral Immunol* **23**, 457-466.

Roberts, J. (2001). Applying PRRSV sequencing to field cases. *Proc Allen D Leman Swine Conf* **28**, 75-78.

Rompato, G., Ling, E., Chen, Z., Van Kruiningen, H. & Garmendia, A. E. (2006). Positive inductive effect of IL-2 on virus-specific cellular responses elicited by a PRRSV-ORF7 DNA vaccine in swine. *Vet Immunol Immunopathol* **109**, 151-160.

Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K. & Levings, M. K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* **212**, 28-50.

Ropp, S. L., Wees, C. E., Fang, Y., Nelson, E. A., Rossow, K. D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J. E., Benfield, D. A. & Faaberg, K. S. (2004). Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. *J Virol* **78**, 3684-3703.

Rossow, K. D., Shivers, J. L., Yeske, P. E., Polson, D. D., Rowland, R. R., Lawson, S. R., Murtaugh, M. P., Nelson, E. A. & Collins, J. E. (1999). Porcine reproductive and respiratory syndrome virus infection in neonatal pigs characterised by marked neurovirulence. *Vet Rec* **144**, 444-448.

Rossow, K. D. (1998). Porcine reproductive and respiratory syndrome. *Vet Pathol* **35**, 1-20.

Rossow, K. D., Benfield, D. A., Goyal, S. M., Nelson, E. A., Christopher-Hennings, J. & Collins, J. E. (1996). Chronological immunohistochemical detection and localization of porcine reproductive and respiratory syndrome virus in gnotobiotic pigs. *Vet Pathol* **33**, 551-556.

Rossow, K. D., Collins, J. E., Goyal, S. M., Nelson, E. A., Christopher-Hennings, J. & Benfield, D. A. (1995). Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. *Vet Pathol* **32**, 361-373.

Rossow, K. D., Bautista, E. M., Goyal, S. M., Molitor, T. W., Murtaugh, M. P., Morrison, R. B., Benfield, D. A. & Collins, J. E. (1994). Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. *J Vet Diagn Invest* **6**, 3-12.

Rowland, R. R. (2010). The interaction between PRRSV and the late gestation pig fetus. *Virus Res* **154**, 114-122.

Rowland, R. R., Steffen, M., Ackerman, T. & Benfield, D. A. (1999b). The evolution of porcine reproductive and respiratory syndrome virus: quasispecies and emergence of a virus subpopulation during infection of pigs with VR-2332. *Virology* **259**, 262-266.

Rowland, R. R. & Yoo, D. (2003). Nucleolar-cytoplasmic shuttling of PRRSV nucleocapsid protein: a simple case of molecular mimicry or the complex regulation by nuclear import, nucleolar localization and nuclear export signal sequences. *Virus Res* **95**, 23-33.

Sagong, M., Park, C.K., Kim, S.H., Lee, K.K., Lee, O.S., du Lee, S., Cha, S.Y. & Lee, C. (2012). Human telomerase reverse transcriptase-immortalized porcine monomyeloid cell lines for the production of porcine reproductive and respiratory syndrome virus. *J Virol Methods* **179**, 26-32.

Scortti, M., Prieto, C., Alvarez, E., Simarro, I. & Castro, J. M. (2007). Failure of an inactivated vaccine against porcine reproductive and respiratory syndrome to protect gilts against a heterologous challenge with PRRSV. *Vet Rec* **161**, 809-813.

Scortti, M., Prieto, C., Simarro, I. & Castro, J. M. (2006). Reproductive performance of gilts following vaccination and subsequent heterologous challenge with European strains of porcine reproductive and respiratory syndrome virus. *Theriogenology* **66**, 1884-1893.

Shi, M., Lam, T. T., Hon, C. C., Hui, R. K., Faaberg, K. S., Wennblom, T., Murtaugh, M. P., Stadejek, T. & Leung, F. C. (2010). Molecular epidemiology of PRRSV: a phylogenetic perspective. *Virus Res* **154**, 7-17.

Shibata, I., Uruno, K. & Mori, M. (1996). Serological property and replication in cell cultures of porcine reproductive and respiratory syndrome viruses isolated in Japan. *J Vet Med Sci* **58**, 805-807.

Shimizu, M., Yamada, S., Murakami, Y., Morozumi, T., Kobayashi, H., Mitani, K., Ito, N., Kubo, M., Kimura, K., Kobayashi, M., Yamamoto, K., Miura, Y., Yamamoto, T. & Watanabe, K. (1994). Isolation of Porcine Reproductive and Respiratory Syndrome (Prrs) Virus from Heko-Heko Disease of Pigs. *Journal of Veterinary Medical Science* **56**, 389-391.

Sidell, S. & Snijder, E.J. (2008). An introduction to Nidoviruses. In Nidoviruses, pp. 433. Edited by S. Perlman, Galagher, T. and Snijder, E.J. Washington DC: ASM Press.

Silva-Campa, E., Flores-Mendoza, L., Resendiz, M., Pinelli-Saavedra, A., Mata-Haro, V., Mwangi, W. & Hernandez, J. (2009). Induction of T helper 3 regulatory cells by

dendritic cells infected with porcine reproductive and respiratory syndrome virus. *Virology* **387**, 373-379.

Snijder, E. J., Brinton, M.A., Faaberg, K.A., Godeny, E.K., Gorbalenya, A.E., McLachlan, N.J., Mengeling, W.L. and Plagemann, P.G.W. (2004). Family Arteriviridae. In virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. Edited by C. M. Fauquet, Mayo, M.A., Manoloff, J., Desselberger, U. and Ball, L.A. London Elsevier/Academic Press.

Snijder, E. J. & Meulenberg, J. J. (1998). The molecular biology of arteriviruses. *J Gen Virol* **79**, 961-979.

Spilman, M. S., Welbon, C., Nelson, E. & Dokland, T. (2009). Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. *J Gen Virol* **90**, 527-535.

Stadejek, T., Oleksiewicz, M. B., Scherbakov, A. V., Timina, A. M., Krabbe, J. S., Chabros, K. & Potapchuk, D. (2008). Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. *Arch Virol* **153**, 1479-1488.

Stadejek, T., Oleksiewicz, M. B., Potapchuk, D. & Podgorska, K. (2006). Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes. *J Gen Virol* **87**, 1835-1841.

Stadejek, T., Stankevicius, A., Storgaard, T., Oleksiewicz, M. B., Belak, S., Drew, T. W. & Pejsak, Z. (2002). Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. *J Gen Virol* **83**, 1861-1873.

Suarez, P., Diaz-Guerra, M., Prieto, C., Esteban, M., Castro, J. M., Nieto, A. & Ortin, J. (1996). Open reading frame 5 of porcine reproductive and respiratory syndrome virus as a cause of virus-induced apoptosis. *J Virol* **70**, 2876-2882.

Sur, J. H., Doster, A. R., Christian, J. S., Galeota, J. A., Wills, R. W., Zimmerman, J. J. & Osorio, F. A. (1997). Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis, and induces germ cell death by apoptosis. *J Virol* **71**, 9170-9179.

Suradhat, S. & Thanawongnuwech, R. (2003). Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. *J Gen Virol* **84**, 2755-2760.

Swenson, S. L., Hill, H. T., Zimmerman, J. J., Evans, L. E., Landgraf, J. G., Wills, R. W., Sanderson, T. P., McGinley, M. J., Brevik, A. K., Ciszewski, D. K. & et al. (1994). Excretion of porcine reproductive and respiratory syndrome virus in semen after experimentally induced infection in boars. *J Am Vet Med Assoc* **204**, 1943-1948.

Takamatsu, H. H., Denyer, M. S., Stirling, C., Cox, S., Aggarwal, N., Dash, P., Wileman, T. E. & Barnett, P. V. (2006). Porcine gammadelta T cells: possible roles on the innate and adaptive immune responses following virus infection. *Vet Immunol Immunopathol* **112**, 49-61.

Takaoka, A. & Yanai, H. (2006). Interferon signalling network in innate defence. *Cell Microbiol* **8**, 907-922.

Terpstra, C., Wensvoort, G. & Pol, J. M. (1991). Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet Q* **13**, 131-136.

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

Thanawongnuwech, R., Thacker, B., Halbur, P. & Thacker, E. L. (2004). Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae. *Clin Diagn Lab Immunol* **11**, 901-908.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S.,

Kitamura, Y., Yan, J. & Gao, G. F. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS ONE* **2**, e526.

Timoney, P. J. & McCollum, W. H. (1993). Equine viral arteritis. *Vet Clin North Am Equine Pract* **9**, 295-309.

Tong, G. Z., Zhou, Y. J., Hao, X. F., Tian, Z. J., An, T. Q. & Qiu, H. J. (2007). Highly pathogenic porcine reproductive and respiratory syndrome, China. *Emerg Infect Dis* **13**, 1434-1346.

Van Breedam, W., Delputte, P. L., Van Gorp, H., Misinzo, G., Vanderheijden, N., Duan, X. & Nauwynck, H. J. (2010). Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. *J Gen Virol* **91**, 1659-1667.

Van Breedam, W., Van Gorp, H., Zhang, J. Q., Crocker, P. R., Delputte, P. L. & Nauwynck, H. J. (2010). The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. *PLoS Pathog* **6**, e1000730.

Van Gorp, H., Van Breedam, W., Delputte, P. L. & Nauwynck, H. J. (2008). Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. *J Gen Virol* **89**, 2943-2953.

Vanhee, M., Van Breedam, W., Costers, S., Geldhof, M., Noppe, Y., & Nauwynck, H. J. (2011). Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use off peptide-specific serum antibodies. *Vaccine* **29**, 4794-4804.

Vanhee, M., Costers, S., Van Breedam, W., Geldhof, M., Van Doorsselaere, J., & Nauwynck, H. J. (2010). A variable region in GP4 of European-type porcine reproductive and respiratory syndrome virus induces neutralizing antibodies against homologous but not heterologous virus strains. *Viral Immunol* **23**, 403-413.

Vanhee, M., Delputte, P. L., Delrue, I., Geldhof, M. F. & Nauwynck, H. J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Vet. Res* **40**, 63.

van Nieuwstadt, A. P., Meulenberg, J. J., van Essen-Zanbergen, A., Petersen-den Besten, A., Bende, R. J., Moormann, R. J. & Wensvoort, G. (1996). Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (Arteriviridae) are structural proteins of the virion. *J Virol* **70**, 4767-4772.

Van Reeth, K., Nauwynck, H., & Pensaert, M. (2001). Clinical effects of experimental dual infections with porcine reproductive and respiratory syndrome virus followed by swine influenza virus in conventional and colostrum-deprived pigs. *J Vet Med B Infect Dis Vet Public Health* **48**, 283-292.

Van Reeth, K., Labarque, G., Nauwynck, H. & Pensaert, M. (1999). Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. *Res Vet Sci* **67**, 47-52.

Van Reeth, K., Nauwynck, H. & Pensaert, M. (1996). Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. *Vet Microbiol* **48**, 325-335.

van Vugt, J. J., Storgaard, T., Oleksiewicz, M. B. & Bøtner, A. (2001). High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. *J Gen Virol* **82**, 2615-2620.

van Woensel, P. A., Liefkens, K. & Demaret, S. (1998a). Effect on viraemia of an American and a European serotype PRRSV vaccine after challenge with European wild-type strains of the virus. *Vet Rec* **142**, 510-512.

van Woensel, P. A., Liefkens, K. & Demaret, S. (1998b). European serotype PRRSV vaccine protects against European serotype challenge whereas an American serotype vaccine does not. *Adv Exp Med Biol* **440**, 713-718.

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., Vandekerckhove, J., Van Damme, J., van Woensel, P. A. & Nauwynck, H. J. (2003). Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. *J Virol* **77**, 8207-8215.

Vashisht, K., Goldberg, T. L., Husmann, R. J., Schnitzlein, W. & Zuckermann, F. A. (2008). Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus. *Vaccine* **26**, 4747-4753

Vezina, S. A., Loemba, H., Fournier, M., Dea, S. & Archambault, D. (1996). Antibody production and blastogenic response in pigs experimentally infected with porcine reproductive and respiratory syndrome virus. *Can J Vet Res* **60**, 94-99.

Villadangos, J. A. & Young, L. (2008). Antigen-presenting properties of plasmacytoid dendritic cells. *Immunity* **29**, 352-361.

Vu, H. L., Kwon, B., Yoon, K. J., Laegreid, W. W., Pattnaik, A. K. & Osorio, F. A. (2011). Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. *J Virol* **85**, 5555-5564.

Wei, Z., Zhang, J., Zhuang, J., Sun, Z., Gao, F. & Yuan, S. (2013). Immunization of pigs with a type 2 modified live PRRSV vaccine prevents the development of a deadly long lasting hyperpyrexia in a challenge study with highly pathogenic PRRSV JX143. *Vaccine* pii: S0264-410X(13)00165-5. doi: 10.1016/j.vaccine.2013.02.012.

Weiland, E., Wieczorek-Krohmer, M., Kohl, D., Conzelmann, K. K. & Weiland, F. (1999). Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. *Vet Microbiol* **66**, 171-186.

Wenhui, L., Zhongyan, W., Guanqun, Z., Zhili, L., JingYun, M., Qingmei, X., Baoli, S., & Yingzuo, B. (2012). Complete genome sequence of a novel variant porcine reproductive and respiratory syndrome virus (PRRSV) strain: evidence for recombination between vaccine and wild-type PRRSV strains. *J Virol* **86**, 9543.

Wensvoort, G., de Kluyver, E. P., Pol, J. M., Wagenaar, F., Moormann, R. J., Hulst, M. M., Bloemraad, R., den Besten, A., Zetstra, T. & Terpstra, C. (1992). Lelystad virus, the cause of porcine epidemic abortion and respiratory syndrome: a review of mystery swine disease research at Lelystad. *Vet Microbiol* **33**, 185-193.

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. & et al. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q* **13**, 121-130.

Wesley, R. D., Mengeling, W. L., Lager, K. M., Vorwald, A. C. & Roof, M. B. (1999). Evidence for divergence of restriction fragment length polymorphism patterns following in vivo replication of porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **60**, 463-467.

Wieringa, R., de Vries, A. A. & Rottier, P. J. (2003). Formation of disulfide-linked complexes between the three minor envelope glycoproteins (GP2b, GP3, and GP4) of equine arteritis virus. *J Virol* **77**, 6216-6226.

Wills, R. W., Doster, A. R., Galeota, J. A., Sur, J. H. & Osorio, F. A. (2003). Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. *J Clin Microbiol* **41**, 58-62.

Wills, R. W., Zimmerman, J. J., Yoon, K. J., Swenson, S. L., Hoffman, L. J., McGinley, M. J., Hill, H. T. & Platt, K. B. (1997). Porcine reproductive and respiratory syndrome virus: routes of excretion. *Vet Microbiol* **57**, 69-81.

Wissink, E. H., Kroese, M. V., van Wijk, H. A., Rijsewijk, F. A., Meulenberg, J. J. & Rottier, P. J. (2005). Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. *J Virol* **79**, 12495-12506.

Wongyanin, P., Buranapraditkun, S., Chokeshai-Usaha, K., Thanawonguwech, R. & Suradhat, S. (2010). Induction of inducible CD4+CD25+Foxp3+ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Immunol Immunopathol* **133**, 170-182.

Wu, Q., Xu, F., Fang, L., Xu, J., Li, B., Jiang, Y., Chen, H. & Xiao, S. (2013). Enhanced immunogenicity induced by an alphavirus replicon-based pseudotyped baculovirus vaccine against porcine reproductive and respiratory syndrome virus. *J Virol Methods* **187**, 251-258.

Wu, W. H., Fang, Y., Rowland, R. R., Lawson, S. R., Christopher-Hennings, J., Yoon, K. J. & Nelson, E. A. (2005). The 2b protein as a minor structural component of PRRSV. *Virus Res* **114**, 177-181.

Xiao, Z., Batista, L., Dee, S., Halbur, P. & Murtaugh, M. P. (2004). The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. *J Virol* **78**, 5923-5933.

Yaeger, M. J., Prieve, T., Collins, J., Christopher-Hennings, J., Nelson, E., Benfield, D. (1993). Evidence for the transmission of porcine reproductive and respiratory syndrome (PRRS) virus in boar semen. *Swine Health Prod* **1**, 7-9.

Yang, L., Frey, M. L., Yoon, K. J., Zimmerman, J. J. & Platt, K. B. (2000). Categorization of North American porcine reproductive and respiratory syndrome viruses: epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. *Arch Virol* **145**, 1599-1619.

Yoon, I. J., Joo, H.S., Christianson , W.T., Morrison, R.B. & Dial, G.D. (1993). Persistent and contact infection in nursery pigs experimentally infected with PRRSV. *J Vet Diagn Invest* **6**, 289-292.

Yoon, K. J., Zimmerman, J. J., Chang, C. C., Cancel-Tirado, S., Harmon, K. M. & McGinley, M. J. (1999). Effect of challenge dose and route on porcine reproductive and respiratory syndrome virus (PRRSV) infection in young swine. *Vet Res* **30**, 629-638.

Yoon, K. J., Wu, L. L., Zimmerman, J. J., Hill, H. T. & Platt, K. B. (1996). Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. *Viral Immunol* **9**, 51-63.

Yoon, K. J., Zimmerman, J. J., Swenson, S. L., McGinley, M. J., Eernisse, K. A., Brevik, A., Rhinehart, L. L., Frey, M. L., Hill, H. T. & Platt, K. B. (1995). Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. *J Vet Diagn Invest* **7**, 305-312.

Yoshii, M., Kaku, Y., Murakami, Y., Shimizu, M., Kato, K. & Ikeda, H. (2005). Genetic variation and geographic distribution of porcine reproductive and respiratory syndrome virus in Japan. *Arch Virol* **150**, 2313-2324.

Zhang, X., Wang, X., Mu, L. & Ding, Z. (2012). Immune responses in pigs induced by recombinant DNA vaccine co-expressing Swine IL-18 and membrane protein of porcine reproductive and respiratory syndrome virus. *Int J Mol Sci* **13**(5): 5715-5728.

Zhou, Y. J., Hao, X. F., Tian, Z. J., Tong, G. Z., Yoo, D., An, T. Q., Zhou, T., Li, G. X., Qiu, H. J., Wei, T. C. & Yuan, X. F. (2008). Highly virulent porcine reproductive and respiratory syndrome virus emerged in China. *Transbound Emerg Dis* **55**, 152-164.

Zimmerman, J., Benfield, D. A., Murtaugh, M. P., Osorio, F., Stevenson, G. W. & Torremorell, M. (2006). Porcine Reproductive and Respiratory Syndrome Virus (Porcine Arterivirus). In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J., (Eds.), Diseases of Swine 9th Edition. Blackwell Science, USA, pp. 387-417.

Zuckermann, F. A., Garcia, E. A., Luque, I. D., Christopher-Hennings, J., Doster, A., Brito, M. & Osorio, F. (2007). Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol* **123**, 69-85.

CHAPTER 2

PROBLEM STATEMENTS & AIMS

PROBLEM STATEMENTS & AIMS

PRRSV causes the economically most important viral syndrome in swine industry: porcine reproductive and respiratory syndrome. It is generally acknowledged that there is an urgent need for a new generation of PRRSV vaccines that are safe, protect against disease and viral spread, deal with the high variability of the virus, and allow differentiation between vaccinated and infected animals.

In 2009, Vanhee et al. demonstrated that, by the use of a controlled inactivation procedure and a suitable adjuvant, an LV-based inactivated PRRSV vaccine could be developed that consistently induces an LV-specific VN antibody response upon 2 vaccinations in naïve piglets. Following a homologous challenge with LV, vaccinated pigs developed an earlier and stronger VN antibody response compared to naïve pigs, and a significant reduction of viremia was observed.

At the start of this thesis, it was unknown whether it was possible to achieve similar results for PRRSV variants that are currently circulating and causing reproductive and respiratory disorders in the field.

The main goal of this thesis was to investigate if this novel method of vaccine preparation can be adapted to currently circulating PRRSV variants and if these vaccines can be improved by using a novel virus production system.

The first study aimed to evaluate the capacity of experimental inactivated autogenous PRRSV vaccines to induce PRRSV-specific (VN) antibody response in naïve pigs and to protect these animals against homologous PRRSV challenge. The efficacy of these vaccines was compared with that of experimental heterologous inactivated vaccines and several commercial inactivated and attenuated vaccines (Chapter 3 part 1).

The second study evaluated the PRRSV-specific humoral immune response in PRRSV-immune (non-) pregnant sows upon booster vaccination with inactivated farm-specific PRRSV vaccines (homologous) and commercial PRRSV vaccines (heterologous), as well a the effects of vaccination on maternal immunity (Chapter 3 part 2).

A third study investigated the influence of the viral production system on inactivated vaccine efficacy. Two recent PRRSV isolates were grown on MARC-145 cells, the

African green monkey kidney cell line routinely used for PRRSV virus production, as well as on $PK15^{Sn\text{-}CD163}$ cells, a recently developed porcine kidney cell line expressing the macrophage-specific PRRSV receptors Sn and CD163 and virus stocks were used for vaccine preparation. The efficacy of these inactivated PRRSV vaccines was evaluated in homologous and heterologous challenge experiments (Chapter 4).

REFERENCES

Vanhee, M., Delputte, P. L., Delrue, I., Geldhof, M. F., Nauwynck, H. J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Vet Res* **40**, 63.

CHAPTER 3

TO TEST AN INACTIVATED VACCINE BASED ON FARM-SPECIFIC PRRSV STRAINS

3.1

COMPARISON OF THE EFFICACY OF AUTOGENOUS INACTIVATED PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) VACCINES WITH THAT OF COMMERCIAL VACCINES AGAINST HOMOLOGOUS AND HETEROLOGOUS CHALLENGES

Marc F Geldhof, Merijn Vanhee, Wander Van Breedam, Jan Van Doorsselaere, Uladzimir U Karniychuk, Hans J Nauwynck

BMC Veterinary Research (2012), 8:182

The porcine reproductive and respiratory syndrome virus (PRRSV) is a rapidly evolving pathogen of swine. At present, there is a high demand for safe and more effective vaccines that can be adapted regularly to emerging virus variants. A recent study showed that, by the use of a controlled inactivation procedure, an experimental BEI-inactivated PRRSV vaccine can be developed that offers partial protection against homologous challenge with the prototype strain LV. At present, it is however not known if this vaccine can be adapted to currently circulating virus variants. In this study, two recent PRRSV field isolates (07V063 and 08V194) were used for BEI-inactivated vaccine production. The main objective of this study was to assess the efficacy of these experimental BEI-inactivated vaccines against homologous and heterologous challenge and to compare it with an experimental LV-based BEI-inactivated vaccine and commercial inactivated and attenuated vaccines. In addition, the induction of challenge virus-specific (neutralizing) antibodies by the different vaccines was assessed.

In a first experiment (challenge with 07V063), vaccination with the experimental homologous (07V063) inactivated vaccine shortened the viremic phase upon challenge with approximately 2 weeks compared to the mock-vaccinated control group. Vaccination with the commercial attenuated vaccines reduced the duration of viremia with approximately one week compared to the mock-vaccinated control group. In contrast, the experimental heterologous (LV)

inactivated vaccine and the commercial inactivated vaccine did not influence viremia. Interestingly, both the homologous and the heterologous experimental inactivated vaccine induced 07V063-specific neutralizing antibodies upon vaccination, while the commercial inactivated and attenuated vaccines failed to do so.

In the second experiment (challenge with 08V194), use of the experimental homologous (08V194) inactivated vaccine shortened viremia upon challenge with approximately 3 weeks compared to the mock-vaccinated control group. Similar results were obtained with the commercial attenuated vaccine. The experimental heterologous (07V063 and LV) inactivated vaccines did not significantly alter viremia. In this experiment, 08V194-specific neutralizing antibodies were induced by the experimental homologous and heterologous inactivated vaccines and a faster appearance post challenge was observed with the commercial attenuated vaccine.

The experimental homologous inactivated vaccines significantly shortened viremia upon challenge. Despite the concerns regarding the efficacy of the commercial attenuated vaccines used on the farms where the field isolates were obtained, use of commercial attenuated vaccines clearly shortened the viremic phase upon challenge. In contrast, the experimental heterologous inactivated vaccines and the commercial inactivated vaccine had no or only a limited influence on viremia. The observation that homologous BEI-inactivated vaccines can provide a more or less standardized, predictable degree of protection against a specific virus variant suggests that such vaccines may prove useful in case virus variants emerge that escape the immunity induced by the attenuated vaccines.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) infection is characterized by reproductive failure in sows, and is associated with respiratory problems in pigs of all ages (Christianson et al., 1992; Christianson et al., 1993; Lager & Mengeling, 1995; Mengeling et al., 1994; Rossow et al., 1994; Terpstra et al., 1991; Van Gucht et al., 2004). With few exceptions, PRRSV is present in a majority of swine-producing countries around the world and gives rise to significant economic losses in the swine industry (Neumann et al., 2005). Based on genetic and antigenic analysis, two PRRSV genotypes are recognized: a European (EU) genotype (prototype: Lelystad virus, LV) (Wensvoort et al., 1991) and a North American (NA) genotype (prototype: VR2332), which share about 55-70% nucleotide homology (Allende et al., 1999). However, a high genetic variability has been demonstrated within both genotypes (Allende et al., 1999; Forsberg et al., 2002; Mateu et al., 2003; Nelsen et al., 1999) and the genetic differences between virus variants are mirrored in different virulence, pathogenicity, immunogenicity, … A recent study by Diaz et al. (2012) showed that infection with different PRRSV strains leads to different virological and immunological outcomes and results in different degrees of homologous and heterologous protection. Another study by Martinez-Lobo and coworkers (2011) reported that different PRRSV isolates differ in their susceptibility to antibody neutralization. Evidently, the high variability of the virus represents a major hurdle for effective PRRSV prevention and control (Meng, 2000). Since PRRSV poses a serious burden on the swine industry worldwide, the need for efficient control measures is high. A variety of PRRS eradication strategies have been described, including total depopulation/repopulation, partial depopulation, segregated early weaning, test and removal and herd closure. Also planned exposure to a farm-specific virus isolate is a common strategy in the United States and Canada (Opriessnig et al., 2007). This last approach is often performed without monitoring and is consequently unreliable in getting the targeted population homogeneously infected in a timely manner. While the above strategies can certainly be useful, it is also clear that efficient PRRSV vaccines are extremely valuable tools to minimize the clinical and economical impact of PRRSV infections. However, the commonly used vaccines, both attenuated and inactivated, are not without their problems. Although attenuated vaccines have the potential to protect animals against viremia, the degree

of protection depends on various factors, including the homology between the vaccine virus and the circulating virus (Labarque et al., 2004). In addition, there are some safety concerns, as the vaccine virus may spread and revert to virulence (Dewey et al., 1999; Mengeling et al., 1999; Nielsen et al., 1997; Nielsen et al., 2001). The commercially available inactivated vaccines are generally safe to use, but do not provide sufficient protection (Nielsen et al., 1997; Nilubol et al., 2004; Zuckermann et al., 2007). In addition, the ability of PRRSV to subvert the host immune system further complicates these matters. At present, it is generally accepted that there is a need for new and safe vaccines that can protect against infection with those virus variants that escape immunity induced by the currently available commercial vaccines. In this context, the use of vaccine virus that is homologous to the PRRSV variants prevalent in the herd seems to be favourable (Labarque et al., 2004). Vanhee et al. (2009) demonstrated that, by use of a controlled inactivation procedure and a suitable adjuvant, an LV-based inactivated PRRSV vaccine can be developed that systematically induces an LV-specific virus-neutralizing (VN) antibody response upon 2 vaccinations in naïve piglets. Following homologous challenge of the vaccinated pigs with LV, animals developed an earlier and strongly elevated VN antibody response and a significant reduction of viremia was observed (Vanhee et al., 2005). Currently however, it is unknown whether it is possible to achieve similar results for PRRSV isolates that are currently causing reproductive or respiratory disorders in the field. Two recent PRRSV isolates, from outbreaks in herds vaccinated with a registered vaccine, were used for autogenous inactivated vaccine development. The main objective of this study was to test the capacity of experimental inactivated autogenous PRRSV vaccines to protect naïve pigs against homologous PRRSV challenge and to compare the efficacy of these vaccines with that of experimental heterologous inactivated vaccines, the commercial vaccine used on the farms, and other commercial inactivated and attenuated vaccines.

MATERIALS AND METHODS

Cells and viruses

Porcine alveolar macrophages (PAMs) were derived from 3-week-old (just weaned) piglets, purchased from a PRRSV- and *Mycoplasma Hyopneumoniae*-negative farm. After isolation, the morphology of PAMs was checked visually via light microscopy.

No specific tests were performed to detect PCV2. PAMs and MARC-145 cells were cultivated as described before (Delrue, 2010).

The Belgian PRRSV isolates used in this study originated from two farms showing clinical signs compatible with PRRS in sows or growing pigs. The two isolates were randomly selected from 19 isolates obtained between 2007 and 2010. At the moment of sampling, sows of both herds were vaccinated with a EU-genotype attenuated vaccine (Porcilis® PRRS). PRRSV isolate 07V063 was isolated from fetal tissue by inoculating tissue suspensions on PAM. This isolate has been used in recent studies by Karniychuk et al. (2011; 2012), describing viral, clinical and pathological data. Similarly, the 08V194 isolate was obtained by inoculating the serum of 14-week-old piglets on PAM. Both isolates were also adapted to MARC-145 cells by repeated passages. For challenge, macrophage-grown stocks were prepared of the isolates 07V063 (2^{nd} passage on PAM) and 08V194 (5^{th} passage on PAM).

For vaccine preparation, MARC-145 cell culture supernatants of 07V063 (2^{nd} passage on PAM + 2 passages on MARC-145), 08V194 (2^{nd} passage on PAM + 4 passages on MARC-145) and LV (2^{nd} passage on PAM + 5 passages on MARC-145), were purified via ultracentrifugation as previously described by Vanhee et al. (2009).

Genome sequencing and phylogenetic analysis

To determine if adaptation to the MARC-145 cell line resulted in mutations in the structural ORFs, ORF2-7 of MARC-145-grown 07V063, 08V194 and LV were sequenced and compared with those of original macrophage-grown 07V063, 08V194 and LV. Sequencing was performed as described before (Delrue et al., 2010). Nucleotide sequences were submitted to Genbank under accession numbers [GenBank: GU737264] (07V063) and [GenBank: GU737265] (08V194).

Amino acid (aa) sequences were subsequently derived and analysed using CLC Free workbench 4. The aa sequences of all structural proteins of MARC-145-grown 07V063, 08V194 and LV were 100% identical to those of the corresponding proteins of original macrophage-grown virus. The clear difference in aa sequence between both 07V063 and 08V194 and the Porcilis® PRRS strain allowed their classification as EU wild-type viruses that are not of vaccine origin.

Virus inactivation and quality control

Purified virus (07V063, 08V194 and LV) was suspended in RPMI 1640 (Invitrogen) to a titer of 10^8 $TCID_{50}$/mL. Subsequently, the virus was inactivated using BEI as described before (Vanhee et al., 2009), and inactivated virus was stored at -70 °C. To confirm that all virus was completely inactivated, a complete vaccine dose of 07V063, 08V194 and LV was inoculated on MARC-145 cells and subsequently passaged twice. As a positive control, MARC-145 cells were inoculated with 1 mL of non-inactivated 07V063, 08V194 or LV. The MARC-145 cells were routinely checked for cytopathic effect (CPE) and ultimately stained for the PRRSV nucleocapsid protein via an immunoperoxidase staining using monoclonal antibody 13E2 (Van Breedam et al., 2011). No CPE or positive nucleocapsid staining was detected in cells that were inoculated with inactivated virus, while clear CPE and nucleocapsid staining were observed in cell cultures that were inoculated with non-inactivated virus.

Since conservation of entry of inactivated virus may serve as a quality control for the preservation of antigenic properties, the effect of BEI inactivation on virus attachment and internalization into macrophages was examined as described previously (Delrue et al., 2009; Vanhee et al., 2009). Non-inactivated virus suspensions were included as positive controls. The entry experiment showed that the binding and internalization kinetics of all BEI-inactivated virus stocks are similar to those observed for the non-inactivated virus stocks.

Pigs and experimental design

Sixty-seven four-week-old piglets were purchased from a PRRSV-negative farm and their PRRSV-seronegative status was confirmed by IPMA upon arrival. The animals were housed in isolation units with HEPA-filtered air and kept during 7 days to allow adaptation to the new conditions. Two experiments were performed (Table 1). General health, appetite and rectal body temperature of the pigs were monitored daily. All animal experiments were approved by the local ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Table 1: Experimental design of vaccination-challenge experiments

Group	Vaccination	Age in weeks	Challenge strain (13 weeks)
Experiment 1 (n)			
CON (6)	Mock	5 and 9	07V063
07V063i (6)	BEI-inactivated 07V063	5 and 9	07V063
LVi (6)	BEI-inactivated LV	5 and 9	07V063
PROi (6)	Progressis®	5 and 9	07V063
PORatt (6)	Porcilis® PRRS	7	07V063
INGatt (6)	Ingelvac® PRRS	7	07V063
Experiment 2 (n)			
CON2 (7)	Mock	5 and 9	08V194
08V194i (6)	BEI-inactivated 08V194	5 and 9	08V194
LVi2 (6)	BEI-inactivated LV	5 and 9	08V194
07V063i2 (6)	BEI-inactivated 07V063	5 and 9	08V194
PORatt2 (6)	Porcilis® PRRS	7	08V194

Vaccination experiment with PRRSV isolate 07V063

Thirty-six pigs were randomly divided into six groups. An oil-in-water (o/w) diluent, normally used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky (Fort Dodge Animal Health), was used as an adjuvant and is further referred to as o/w Suvaxyn. A first group (group CON, n = 6 pigs) served as a mock-vaccinated control group and received 1 mL RPMI 1640 in 1 mL o/w Suvaxyn intramuscularly at 5 and 9 weeks of age. Three other groups were vaccinated twice intramuscularly at 5 (primo vaccination) and 9 (booster vaccination) weeks of age. Group 07V063i (n = 6 pigs) was vaccinated with 1 mL BEI-inactivated MARC-145-grown 07V063 (10^8 $TCID_{50}$) in 1 mL o/w Suvaxyn and group LVi (n = 6 pigs) was vaccinated with 1 mL BEI-inactivated MARC-145-grown LV (10^8 $TCID_{50}$) in 1 mL o/w Suvaxyn. Group PROi (n = 6 pigs) received 2 mL of a commercial European type inactivated PRRSV vaccine (Progressis®, Merial, strain P120: min 2,5 log IF Units). Groups PORatt (n = 6 pigs) and INGatt (n = 6 pigs) were vaccinated once intramuscularly with the European type attenuated vaccine (Porcilis® PRRS, Intervet, 10^4 $TCID_{50}$/2 mL) and

the American type attenuated vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim, $10^{4.9}$ $TCID_{50}$/2 mL), respectively, at the age of 7 weeks. At 13 weeks of age, all pigs were challenged intranasally with PRRSV 07V063 (10^6 $TCID_{50}$) in phosphate buffered saline (PBS) (2,5 ml per nostril). Blood samples were taken by jugular venipuncture weekly after (primo) vaccination and at 0, 1, 3, 5, 7, 10, 14, 21, 28, 35 and 42 dpc. Serum was collected and stored at -70 °C. Serum samples for IPMA and VN antibody detection were incubated for 30 min at 56 °C prior to freezing.

Vaccination experiment with PRRSV isolate 08V194

In a second experiment, 31 piglets were randomly assigned to five treatment groups. Group CON2 (*n* = 7 pigs) served as a mock-vaccinated control group and received 1 mL RPMI 1640 in 1 mL o/w Suvaxyn intramuscularly at 5 and 9 weeks of age. Three other groups were vaccinated twice intramuscularly at 5 (primo vaccination) and 9 (booster vaccination) weeks of age. Group 08V194i (*n* = 6 pigs) was vaccinated with 1 mL BEI-inactivated MARC-145-grown 08V194 (10^8 $TCID_{50}$) in 1 mL o/w Suvaxyn, group LVi2 (*n* = 6 pigs) was vaccinated with 1 mL BEI-inactivated MARC-145-grown LV (10^8 $TCID_{50}$) in 1 mL o/w Suvaxyn and group 07V063i2 (*n* = 6 pigs) was vaccinated with 1 mL BEI-inactivated MARC-145-grown 07V063 (10^8 $TCID_{50}$) in 1 mL o/w Suvaxyn. At 7 weeks of age, pigs of group PORatt2 (*n* = 6 pigs) were vaccinated intramuscularly with Porcilis® PRRS at a dose of 10^4 $TCID_{50}$ per pig. PRRSV isolate 08V194 at a dose of 10^6 $TCID_{50}$ was used to inoculate all pigs intranasally (2,5 ml per nostril) at the age of 13 weeks. The same experimental design was used as in the first experiment.

Virus titration and serological examinations

Virus titers in serum were determined by virus titration on PAM following a standard procedure (Labarque et al., 2000). 24-h cultivated PAM were inoculated with 10-fold dilution series of the serum samples. 72 hours post inoculation, cells were fixed and an immunoperoxidase staining with monoclonal antibody 13E2 against the PRRSV nucleocapsid protein was performed to visualize infection in the cells (Van Breedam et al., 2011). The titers were calculated as described by Reed and Muench (1938) and expressed as $TCID_{50}$/mL. To check the sensitivity of the PAM, all cell batches were assayed in virus titrations using a PRRSV stock (LV) with a known virus titer.

Serum samples were examined for the presence of PRRSV-specific antibodies using an IPMA as described by Labarque et al. (2000). To detect antibodies against 07V063 (1st experiment), an IPMA was performed on 07V063-infected MARC-145 cells. To detect antibodies against 08V194 (2nd experiment), an IPMA was performed on 08V194-infected MARC-145 cells. VN antibodies were detected by seroneutralization assays on MARC-145 cells using the respective PRRSV challenge isolate. Each serum sample was tested in duplicate. Briefly, serum samples were twofold serially diluted and an equal volume of a PRRSV 07V063 (2nd passage on PAM + 2 passages on MARC-145) or 08V194 (2nd passage on PAM + 4 passages on MARC-145) suspension (titer 2 x 10^3 $TCID_{50}$/ mL) was added to each dilution. After mixing, the plates were incubated at 37 °C for 1 h and 50 µl of the mixture was subsequently transferred to confluent monolayers of MARC-145 cells in 96-well plates. Cells were screened for 7 days after inoculation and the neutralization titer of the sera was recorded as the reciprocal of the highest dilution that inhibited CPE in 50% of the inoculated wells. To check the sensitivity to PRRSV infection of different passages of MARC-145 cells, control titrations using PRRSV stocks (isolate 07V063 and isolate 08V194) with a known virus titer were performed in parallel with each neutralization assay.

Statistical analysis

Antibody titers and virus titers were analyzed by Kruskall-Wallis test, followed by Dunn's multiple comparisons test to determine significant differences with the control groups at different time points. Samples, that tested negative in IPMA, VN or virus isolation were consequently given a numerical value of 0.0. A two-tailed Fisher's exact test was used to determine significant differences between the number of viremic animals in the vaccinated groups and the control groups at different time points. An overall *p* value of 0.05 was taken as the level of statistical significance. All statistical analyses were performed using GraphPad Prism version 5.0a (GraphPad Software, San Diego, California, USA).

RESULTS

Vaccination experiment with PRRSV isolate 07V063

Clinical examination

All animals remained in good health after they were vaccinated. No local or systemic vaccine side effects were noted throughout the trial period. No pigs died during the entire experimental period. Body temperatures fluctuated in all groups and statistically significant differences were not detected. Challenge with PRRSV isolate 07V063 induced moderate fever (higher than 39.5 °C, but not higher than 40.6°C) within 10 days post infection in 32 out of 36 inoculated pigs. The 4 remaining animals did not develop fever. By 11 days post challenge, fever had disappeared in all animals.

Viremia

Upon challenge, all animals became viremic. In the adjuvant control group (group CON), viremia was detected from day 1 after the challenge (3 pigs out of 6) and peaked around 10 days post challenge (dpc), with a mean virus titer of 3.6 $\log_{10}$ $TCID_{50}$/mL. Viremia had cleared in all animals by 5 weeks post challenge (Fig. 1, CON). In the binary ethyleneimine (BEI) inactivated 07V063 group (group 07V063i), all animals became viremic, but the peak viremia occurred earlier (day 5) and was lower (2.9 $\log_{10}$ $TCID_{50}$/mL). From 10 dpc, virus was no longer detected in the serum of any of the animals, but one animal was again viremic at day 21 post challenge (Fig. 1, 07V063i). The mean viral titer in the serum was significantly reduced compared to the control group at days 10 and 14 ($p<0.05$). In group 07V063i, a significantly lower number of viremic piglets was observed compared to group CON on days 10 and 14 post challenge ($p<0.05$). In the BEI-inactivated LV vaccinated group (group LVi), all animals became viremic after challenge, with a peak viremia of 3.6 $\log_{10}$ $TCID_{50}$/mL on day 7. Four weeks post challenge, virus was not found anymore in the serum of any of the 6 pigs (Fig. 1, LVi). The virus titers were not significantly lower than those of group CON. In the group vaccinated with Progressis® (group PROi), viremia was detected in all animals, with a peak of 3.6 $\log_{10}$ $TCID_{50}$/mL around 10-14 dpc. The viremic phase showed a similar pattern as in group CON and virus titers were not significantly reduced. Viremia disappeared in all animals by 5 weeks after challenge

(Fig. 1, PROi). In the group vaccinated with a single shot of Porcilis® PRRS (group PORatt) and the group vaccinated with one dose of Ingelvac® PRRS (group INGatt), a partial reduction in viremia was seen. Viremia peaked at 5 dpc with average titers of 3.3 $\log_{10}$ $TCID_{50}$/mL (group PORatt) and 3.2 $\log_{10}$ $TCID_{50}$/mL (group INGatt) (Fig. 1, PORatt and INGatt). No significant differences in mean virus titers were detected at any time-point between groups PORatt, INGatt and CON. Only at 5 weeks after challenge, all animals of these groups were consistently virus negative. Taking all data on viremia together, group 07V063i was the only group that showed a significantly shortened viremia and a significant decrease in the number of viremic piglets compared to the mock-vaccinated control group.

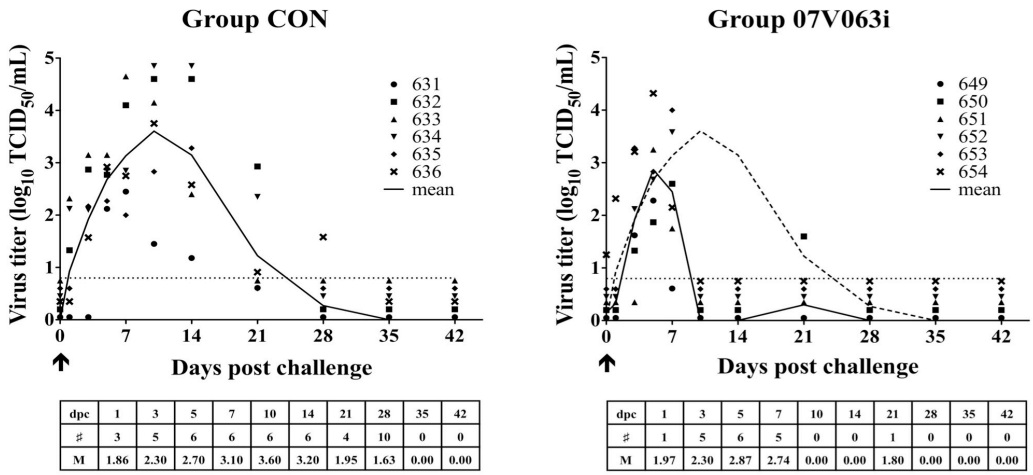
07V063-specific antibodies

In group CON, virus-specific antibodies were not detected before challenge (Fig. 2, CON). At 7 dpc, antibodies could be detected in all animals of this control group. In 2 animals of group 07V063i, antibodies could already be detected at 3 weeks after the primo vaccination. The remaining animals within this group became seropositive after booster vaccination. From the first week after booster vaccination until 10 days after challenge, antibody titers in this group remained significantly higher than in group CON ($p<0.05$) (Fig. 2, 07V063i). In group LVi, virus-specific antibodies against 07V063 were detected from 2 weeks after primo vaccination and all animals seroconverted after booster vaccination. Virus-specific antibody titers were significantly higher in group LVi compared to group CON from 7 days post booster vaccination until 21 dpc and at 42 dpc ($p<0.05$) (Fig. 2, LVi). In group PROi, one animal became seropositive at 1 week after booster vaccination, while the remaining animals did not show antibodies before challenge (Fig. 2, PROi). Post challenge, the course of the antibody response of this group was similar as in group CON. In group PORatt, virus-specific antibodies were detected in 3 out of 6 pigs at 2 weeks after vaccination (Fig. 2, PORatt). At 3 weeks post vaccination, all animals were seropositive. Virus-specific antibodies remained present during the entire experiment, and the antibody titers were significantly higher compared to group CON from 21 days post vaccination until 21 dpc ($p<0.05$). In group INGatt, virus-specific antibodies were found in 3 out of 6 pigs at 2 weeks after vaccination (Fig. 2, INGatt). One week later, all pigs had seroconverted and remained seropositive until the end of

the experiment. Antibody titers were significantly higher compared to group CON from 21 days post vaccination till 5 dpc. Taken together, the courses of the IPMA antibody titers in all groups were similar to those described in other studies (Vanhee et al., 2009; Zuckermann et al., 2007).

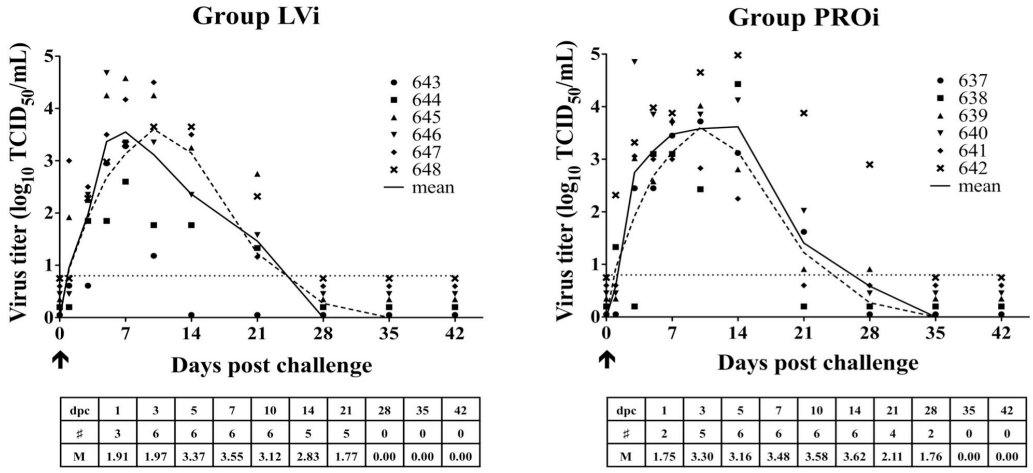
07V063-specific virus-neutralizing antibodies

In group CON, VN antibodies were not detected until 5 weeks post challenge. Even at 6 weeks post challenge, not all the animals from this group were positive for VN antibodies (Fig. 3, CON). In group 07V063i, 07V063-specific VN antibodies were already detected upon booster vaccination. The mean VN antibody titer decreased immediately post challenge, but increased again 10 days after infection. Some animals had no or undetectable VN antibodies in the period between 2 weeks before and 10 dpc, but after this period, VN antibodies were detected in all animals. The mean VN antibody titer was significantly higher compared to group CON in the period between 1 week after booster vaccination and 5 weeks post challenge, reaching mean values ranging from 1.1-4.2 $\log_2$ ($p<0.05$) (Fig. 3, 07V063i). A similar pattern was observed in group LVi: 07V063-neutralizing antibodies were already detected at 1 week after booster vaccination. VN antibody titers initially decreased post challenge and increased again from 10 dpc. Some animals turned negative for VN antibodies in the period between 1 week before and 10 days after the challenge, but after this period, VN antibodies were detected in all 6 animals. Remarkably, one animal in this group did not show neutralizing antibodies earlier than 4 weeks post challenge. The VN antibody titers were significantly higher compared to the control group at 1 and 2 weeks after booster vaccination and at 10 and 14 dpc, reaching mean values of 3.5, 3.1, 1.9 and 2.3 $\log_2$, respectively ($p<0.05$) (Fig. 3, LVi). Pigs that were vaccinated with Progressis® (two shots), Porcilis® PRRS (single shot) and Ingelvac® PRRS (single shot) showed a roughly similar VN antibody response as the animals in the control group. In the Porcilis® vaccinated group, a slight increase of VN antibodies was noticed at 5 weeks post challenge, but there were no significant differences with group CON (Fig. 3, PORatt). In summary, both BEI-inactivated vaccines induced a strong 07V063-specific VN antibody response after booster vaccination, while the commercial vaccines, both inactivated and attenuated (EU or NA genotype), did not induce a VN antibody response against 07V063.



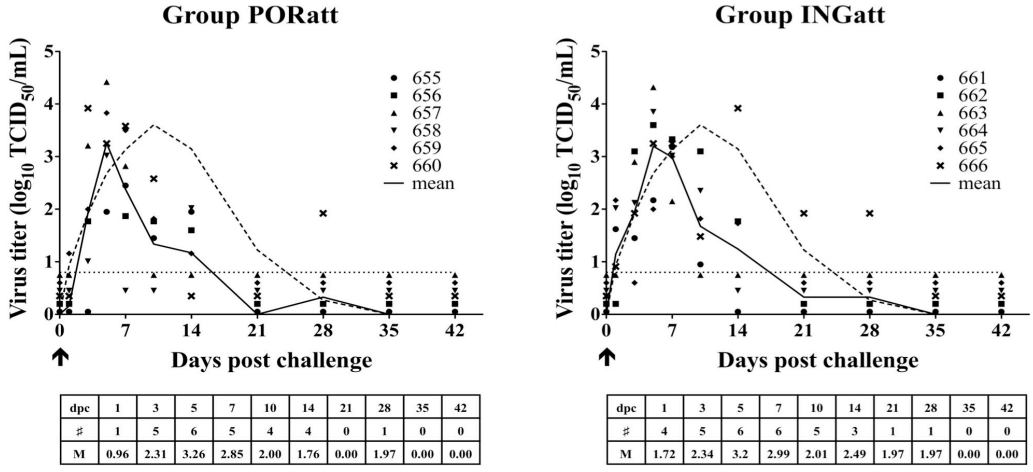
dpc	1	3	5	7	10	14	21	28	35	42
#	3	5	6	6	6	6	4	10	0	0
M	1.86	2.30	2.70	3.10	3.60	3.20	1.95	1.63	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	1	5	6	5	0	0	1	0	0	0
M	1.97	2.30	2.87	2.74	0.00	0.00	1.80	0.00	0.00	0.00



dpc	1	3	5	7	10	14	21	28	35	42
#	3	6	6	6	6	5	5	0	0	0
M	1.91	1.97	3.37	3.55	3.12	2.83	1.77	0.00	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	2	5	6	6	6	6	4	2	0	0
M	1.75	3.30	3.16	3.48	3.58	3.62	2.11	1.76	0.00	0.00



dpc	1	3	5	7	10	14	21	28	35	42
#	1	5	6	5	4	4	0	1	0	0
M	0.96	2.31	3.26	2.85	2.00	1.76	0.00	1.97	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	4	5	6	6	5	3	1	1	0	0
M	1.72	2.34	3.2	2.99	2.01	2.49	1.97	1.97	0.00	0.00

Figure 1. Serum-virus titers after challenge for group CON (Mock-vaccinated control), 07V063i (BEI-inactivated 07V063), LVi (BEI-inactivated LV), PROi (Progressis®), PORatt (Porcilis® PRRS) and INGatt (Ingelvac® PRRS MLV). Virus titers in serum (log_{10} $TCID_{50}$/mL) were determined by virus titration on PAM, followed by immunoperoxidase staining for the PRRSV nucleocapsid protein. ↑ = challenge. Symbols represent individual animals and solid lines represent mean virus titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON. The dotted line marks the detection limit for virus titration. Mentioned in the table: # = the number of viremic animals in the different groups at different time points. M = mean virus titer of all viremic animals in the group at different time points.

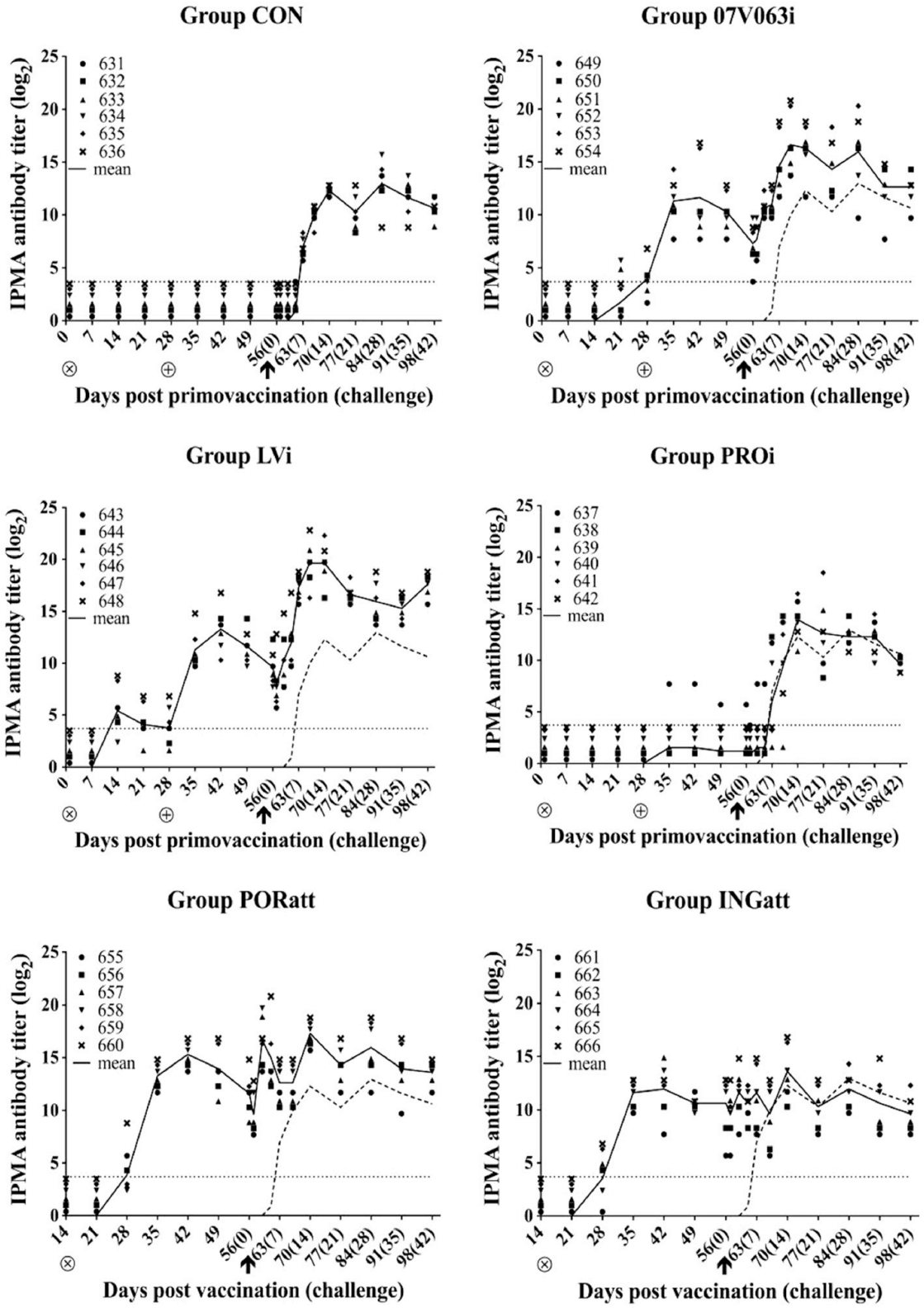


Figure 2. PRRSV-specific IPMA antibody titers (log_2) after vaccination and challenge for group CON (Mock-vaccinated control), 07V063i (BEI-inactivated 07V063), LVi (BEI-inactivated LV), PROi (Progressis®), PORatt (Porcilis® PRRS) and INGatt (Ingelvac® PRRS MLV). ⊗ = primo vaccination; ⊕ = booster vaccination; ↑ = challenge. Symbols represent individual animals and solid lines represent mean IPMA titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON. The dotted line marks the detection limit for the IPMA test.

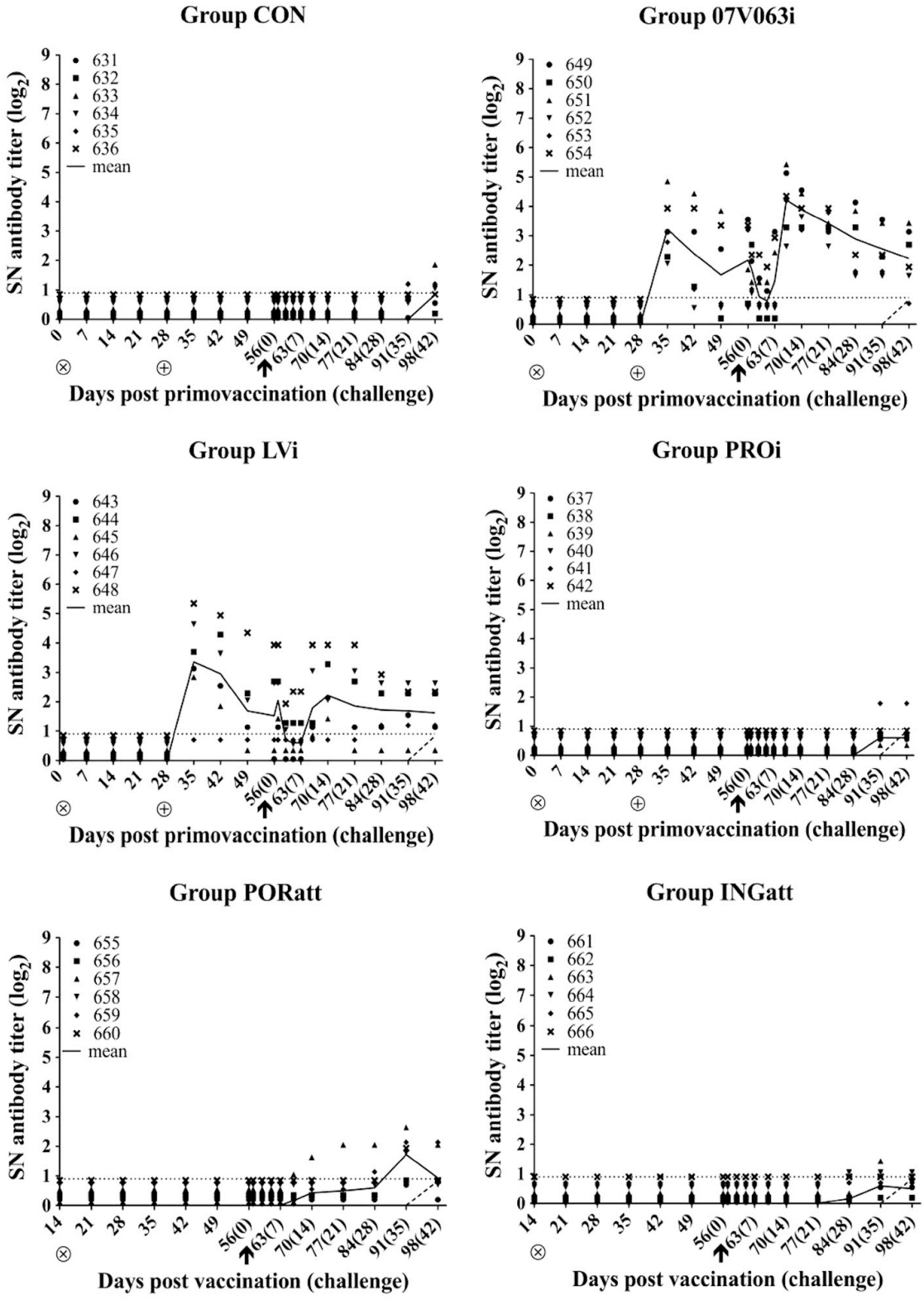


Figure 3. PRRSV-neutralizing antibody titers (log_2) after vaccination and challenge for group CON (Mock-vaccinated control), 07V063i (BEI-inactivated 07V063), LVi (BEI-inactivated LV), PROi (Progressis®), PORatt (Porcilis® PRRS) and INGatt (Ingelvac® PRRS MLV). ⊗ = primo vaccination; ⊕ = booster vaccination; ↑ = challenge. Symbols represent individual animals and solid lines represent mean SN titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON. The dotted line marks the detection limit for the SN test.

Vaccination experiment with PRRSV isolate 08V194

Clinical examination

All animals remained in good health after they were vaccinated. No local or systemic vaccine side effects were noted throughout the trial period. One pig of group PORatt2 died at day 84 at the moment of blood collection. The daily rectal temperatures varied in all groups and no statistically significant differences were observed. Challenge with PRRSV isolate 08V194 induced moderate fever (higher than 39.5 °C, but not higher than 40.4°C) within 7 days post infection in 24 out of 31 inoculated pigs. The 7 remaining animals did not develop fever. By 8 days post challenge, fever had disappeared in all animals.

Viremia

Upon challenge, all animals became viremic. In the control group (group CON2), a maximum mean virus titer of 3.8 $\log_{10}$ $TCID_{50}$/mL was reached at 10 dpc. Subsequently, a decline in virus titer was observed and virus was no longer detectable in the serum at 4, 5 or 6 weeks after challenge, depending on the animal. Still, 1 piglet remained virus positive till 6 weeks post challenge (Fig. 4, CON2). In the BEI-inactivated 08V194 vaccinated group (group 08V194i), the viremic peak at day 5 was not reduced compared to group CON2, but the mean virus titer at day 14 was significantly reduced ($p<0.05$) and from 21 dpc, virus could no longer be detected in any of the piglets (Fig. 4, 08V194i). The number of viremic piglets in group 08V194i was significantly lower compared to group CON2 on day 21 and 28 post challenge ($p<0.05$). Mean virus titers in the group vaccinated with BEI-inactivated LV virus (group LVi2) were comparable to those in group CON2, reaching 3.0 $\log_{10}$ $TCID_{50}$/mL at 10 dpc, and no significant differences could be detected at any time-point between group LVi2 and group CON2. For 3 animals of this group, virus was cleared from the blood at 3 weeks, for 2 others at 4 weeks and in the remaining animal at 5 weeks post challenge (Fig. 4, LVi2). In the BEI-inactivated 07V063 vaccinated group (group 07V063i2), viremia was detected in all animals, with a peak around 5-10 dpc. The viremic phase showed a similar pattern as for group LVi2 and viremia was also not significantly reduced compared to group CON2. Viremia disappeared in all animals by 5 weeks after challenge (Fig. 4, 07V063i2). The mean virus titer in the

group vaccinated with Porcilis® PRRS (group PORatt2) reached 2.7 $\log_{10}$ $TCID_{50}$/mL at 3 days and 2.5 $\log_{10}$ $TCID_{50}$/mL at 5 dpc, but virus titers were not significantly different from those in group CON2 at these time points. At later time points however, virus titers were significantly reduced compared to group CON2 ($p<0.05$). Moreover, viremia in group PORatt2 was already cleared at 10 dpc for 3 animals and at 28 dpc, all animals were negative (Fig. 4, PORatt2). From 14 till 28 dpc, the total number of viremic animals in group PORatt2 was significantly lower than in group CON2 ($p<0.05$). In summary, groups 08V194i and PORatt2 showed a significantly shortened viremia and a significant decrease in the number of viremic piglets compared to the mock-vaccinated control group, while no such effect was seen in groups LVi2 and 07V063i2.

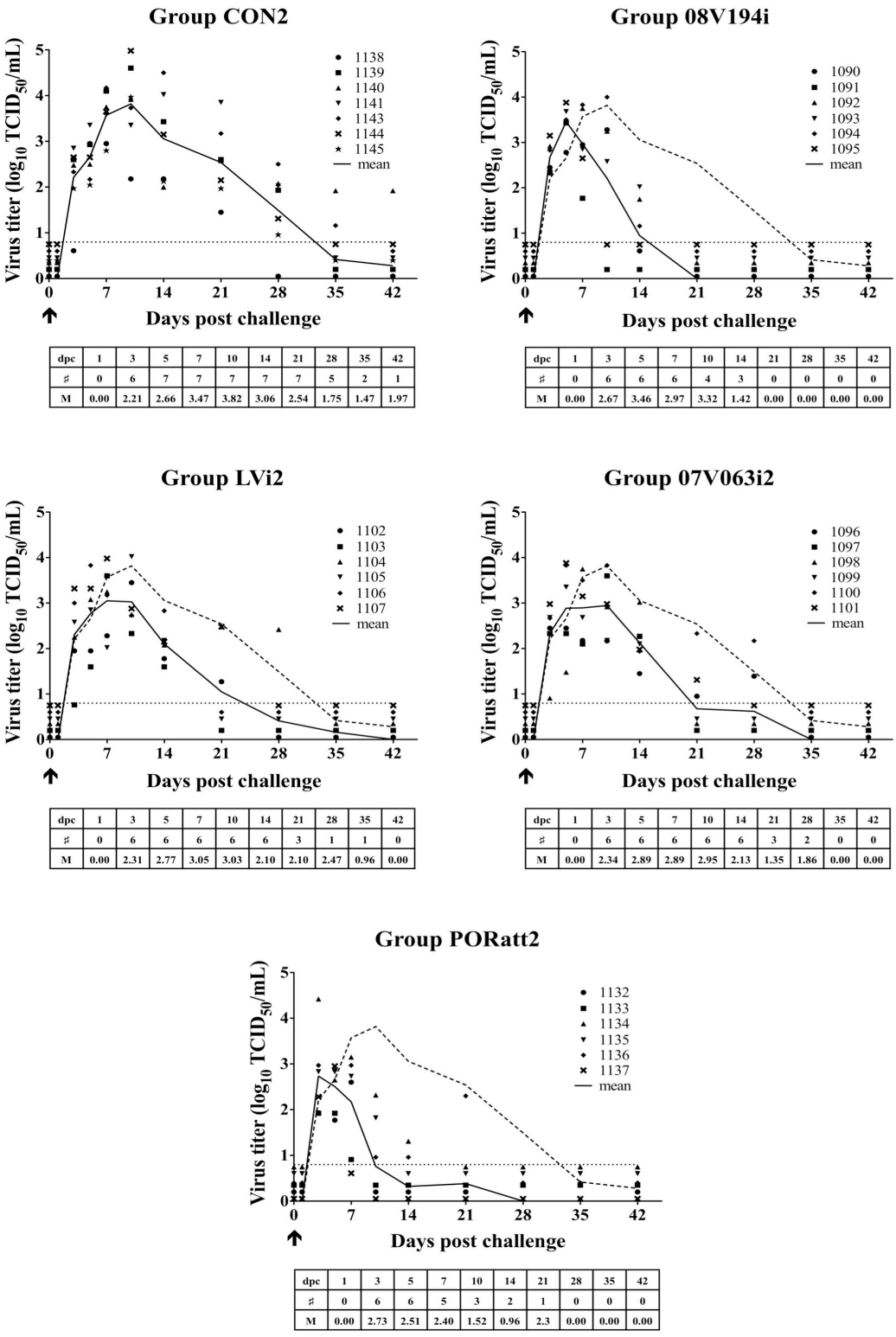
08V194-specific antibodies

All CON2 animals had virus-specific serum antibodies starting from 7 dpc (Fig. 5, CON2). All 6 animals of group 08V194i seroconverted at 2 or 3 weeks after the first vaccination. Similarly, all animals of group LVi2 showed virus-specific antibodies 2 weeks after the first vaccination. In group 07V063i2, 08V194-specific antibodies were detected from 2 weeks after primo vaccination and all animals seroconverted after booster vaccination. Antibody titers in all 3 vaccinated groups were significantly higher compared to group CON2 from 1 week after booster vaccination up till 21 dpc ($p<0.05$) (Fig. 5, 08V194i, LVi2 and 07V063i2). After 21 dpc, mean antibody titers in groups 08V194i, LVi2 and 07V063i2 remained higher compared to the control group, although differences were not significant. In group PORatt2, all pigs showed positive antibody titers at 2 weeks after vaccination; the antibody titers were significantly higher compared to group CON2 starting from 2 weeks after vaccination up till 3 weeks post challenge ($p<0.05$) (Fig. 5, PORatt2). In summary, the course of the IPMA antibody titers in all groups were similar to those described in previous studies and the former experiment in this study (Vanhee et al., 2009; Zuckermann et al., 2007).

08V194-specific virus-neutralizing antibodies

Starting from 21 dpc, 3 pigs of group CON2 showed a VN antibody titer and by 35 dpc, VN antibodies had appeared in all mock-vaccinated pigs (Fig. 6, CON2). All six

pigs of group 08V194i showed high VN antibody titers at 1 week after the booster vaccination and this remained so until the end of the experiment (Fig. 6, 08V194i). VN antibody titers were significantly higher in group 08V194i compared to group CON2 from 1 week after booster vaccination until 5 weeks post challenge, with mean values ranging from 3.2-6.2 $\log_2$ ($p<0.05$). All animals of group LVi2 seroconverted for VN antibodies at least once within 3 weeks after booster vaccination, but VN antibody titers remained low and were only significantly higher than group CON2 at 14 dpc, reaching a mean value of 2.6 $\log_2$ ($p<0.05$) (Fig. 6, LVi2). A similar pattern as in group 08V194i was observed in group 07V063i2, where 08V194-neutralizing antibodies could already be detected at 1 week after booster vaccination. Two animals turned negative for VN antibodies in the period between 2 weeks post booster vaccination and 10 dpc, but after this period, VN antibodies were consistently detected in all 6 animals. The mean VN antibody titer in group 07V063i2 was significantly higher compared to group CON2 in the period between 1 week after booster vaccination and 4 weeks post challenge (except for time-point 5 dpc), reaching mean values ranging from 2.1-4.7 $\log_2$ ($p<0.05$) (Fig. 6, 07V063i2). None of the animals in group PORatt2 showed 08V194-specific VN antibodies before challenge, but VN antibodies already appeared between 5 and 10 dpc. The mean VN antibody titer in group PORatt2 was slightly but not significantly higher compared to group CON2 between 7 and 14 dpc, reaching a maximum of 1.6 $\log_2$ at 14 dpc (Fig. 6, PORatt2). In summary, both BEI-inactivated 08V194 (homologous) and 07V063 (heterologous) vaccines induced a strong 08V194-specific VN antibody response upon booster vaccination, while this was not the case for the heterologous BEI-inactivated LV vaccine and the commercial attenuated vaccine.



dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	7	7	7	7	7	5	2	1
M	0.00	2.21	2.66	3.47	3.82	3.06	2.54	1.75	1.47	1.97

dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	6	6	4	3	0	0	0	0
M	0.00	2.67	3.46	2.97	3.32	1.42	0.00	0.00	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	6	6	6	6	3	1	1	0
M	0.00	2.31	2.77	3.05	3.03	2.10	2.10	2.47	0.96	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	6	6	6	6	3	2	0	0
M	0.00	2.34	2.89	2.89	2.95	2.13	1.35	1.86	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	6	5	3	2	1	0	0	0
M	0.00	2.73	2.51	2.40	1.52	0.96	2.3	0.00	0.00	0.00

Figure 4. Serum-virus titers after challenge for group CON2 (Mock-vaccinated control), 08V194i (BEI-inactivated 08V194), LVi2 (BEI-inactivated LV), 07V063i2 (BEI-inactivated 07V063) and PORatt2 (Porcilis® PRRS). Virus titers in serum (log_{10} $TCID_{50}$/mL) were determined by virus titration on PAM, followed by immunoperoxidase staining for the PRRSV nucleocapsid protein. ↑ = challenge. Symbols represent individual animals and solid lines represent mean virus titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON2. The dotted line marks the detection limit for virus titration. Mentioned in the table: # = the number of viremic animals in the different groups at different time points. M = mean virus titer of all viremic animals in the group at different time points.

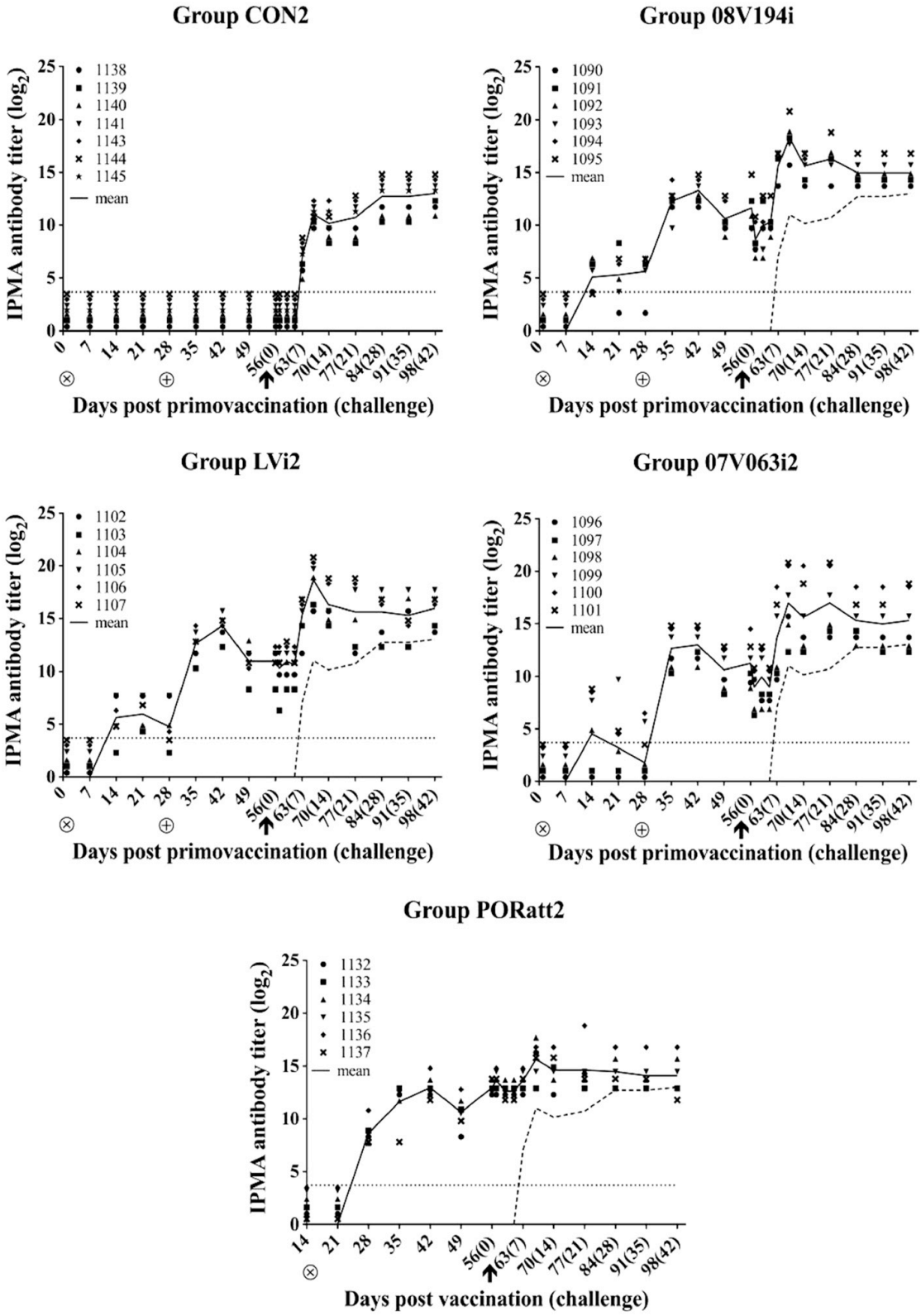


Figure 5. PRRSV-specific IPMA antibody titers (log_2) after vaccination and challenge for group CON2 (Mock-vaccinated control), 08V194i (BEI-inactivated 08V194), LVi2 (BEI-inactivated LV), 07V063i2 (BEI-inactivated 07V063) and PORatt2 (Porcilis® PRRS). ⊗ = primo vaccination; ⊕ = booster vaccination; ↑ = challenge. Symbols represent individual animals and solid lines represent mean IPMA titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON2. The dotted line marks the detection limit for the IPMA test.

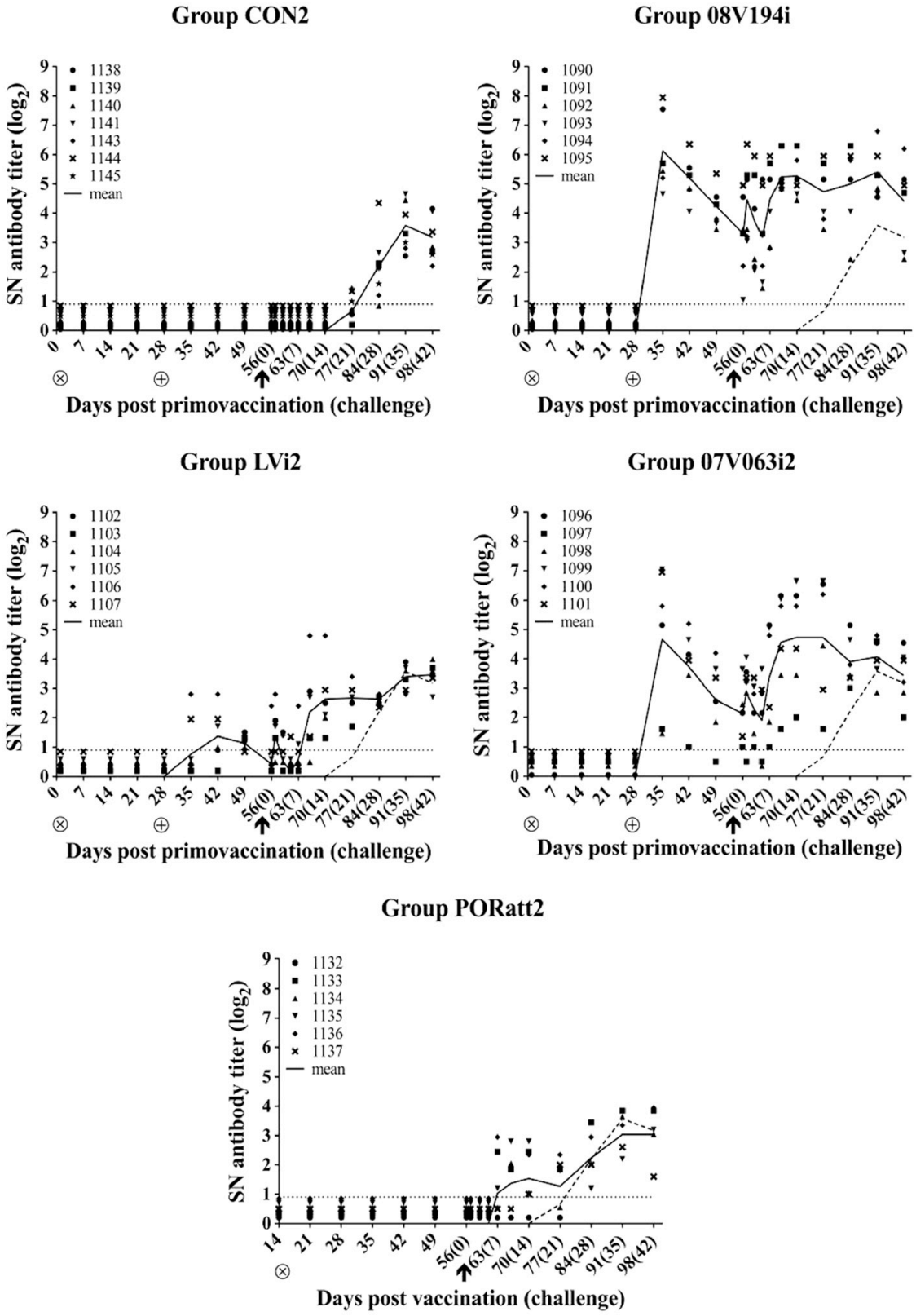


Figure 6. PRRSV-neutralizing antibody titers (log_2) after vaccination and challenge for group CON2 (Mock-vaccinated control), 08V194i (BEI-inactivated 08V194), LVi2 (BEI-inactivated LV), 07V063i2 (BEI-inactivated 07V063) and PORatt2 (Porcilis® PRRS). ⊗ = primo vaccination; ⊕ = booster vaccination; ↑ = challenge. Symbols represent individual animals and solid lines represent mean SN titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON2. The dotted line marks the detection limit for the SN test.

DISCUSSION

PRRSV causes severe reproductive disorders in sows and boars and is associated with the porcine respiratory disease complex. The virus is difficult to control and has become endemic in many major swine-producing countries, leading to tremendous economic losses worldwide (Neumann et al., 2005). To control the disease, several commercial attenuated and inactivated vaccines are currently available. However, when used in the field, these vaccines have met with variable degrees of success. Reported outbreaks of clinical PRRS in vaccinated pigs have led to doubts about the efficacy of currently available vaccines (Thanawongnuwech & Suradhat, 2010). New vaccination strategies are needed to achieve the goals of local and regional elimination of PRRSV and it is generally accepted that a continuous update of vaccine strains is necessary to reach an acceptable level of protection in the field, even within geographical areas of limited size. A recent study by Vanhee et al. (2009) showed that a PRRSV LV-based BEI-inactivated vaccine induces LV-specific VN antibodies in PRRSV-negative animals and offers partial protection upon homologous challenge. In that study, it was however not assessed if such a vaccine can be adapted to field variants of PRRSV that are genetically and antigenically divergent from the currently used vaccine strains. The main objective of the current study was to assess the efficacy of experimental BEI-inactivated vaccines, based on recent PRRSV field isolates (07V063 and 08V194), against homologous and heterologous challenge. A commercial inactivated (Progressis®) and two commercial attenuated (Porcilis® PRRS and Ingelvac® PRRS MLV) PRRSV vaccines were included in the study and served as a reference. Vaccine efficacy was assessed by evaluating the viremia upon challenge – a factor directly linked with viral pathogenesis and spread.

The 07V063- and 08V194-based inactivated PRRSV vaccines were effective in partially protecting naïve pigs upon homologous challenge. They shortened viremia with 2 (07V063) and 3 (08V194) weeks compared to the viremic phase in the respective mock-vaccinated groups, which lasted roughly 1 month. BEI-inactivated LV vaccines were included to assess the impact of strain variability on vaccine efficacy. We found no reduction in 07V063 viremia after the use of an inactivated LV-based vaccine and only a non-significant reduction of viremia upon challenge with 08V194. Similarly, a 07V063-based BEI-inactivated PRRSV vaccine did not significantly reduce viremia upon challenge with the 08V194 isolate. The Progressis®

vaccine did not provide any virological protection, since viremia was observed for 4 weeks upon challenge with the 07V063 isolate. This is in line with the results from previous studies, showing that the commercial inactivated vaccines appear not to influence viremia, even in nearly homologous conditions (Nielsen et al., 1997; Scortti et al., 2007; Zuckermann et al., 2007). Vaccination with the EU-genotype attenuated vaccine reduced the duration of viremia upon challenge with 07V063 with approximately one week. In animals challenged with 08V194, this vaccine shortened viremia from 5 to 2 weeks. The NA-genotype attenuated vaccine reduced viremia in 07V063-challenged animals with approximately one week. Hence, despite the concerns regarding the efficacy of the attenuated vaccine used on both farms, the results of our study indicate that the use of this vaccine in PRRS-naïve pigs can clearly limit viremia. These results are in line with earlier studies published by Cano et al. (2007) and Scortti et al. (2006), showing that attenuated vaccines can be successful in controlling and reducing clinical disease upon homologous and heterologous challenge.

In the field, PRRSV vaccination is mainly performed in sows. Therefore, we reasoned it would also be interesting to assess the antibody response induced by the vaccines, since maternal antibodies play a pivotal role in the passive (colostral) immunity that protects piglets during their first weeks of life (Nechvatalova et al., 2011). Although resolution of PRRSV infection is not always directly correlated with the neutralizing antibody response (Diaz et al., 2006), there is ample evidence that neutralizing antibodies can facilitate virus clearance and, when present in sufficient amounts, may even provide a sterilizing immunity (Diaz et al., 2006; Labarque et al., 2000; Lopez & Osorio, 2004; Lopez et al., 2007). IPMA and SN tests were performed to evaluate the capacity of the vaccines to induce or prime a challenge virus-specific (neutralizing) antibody response.

Vaccination with BEI-inactivated 07V063 or 08V194 vaccines consistently induced sizable titers of homologous PRRSV-neutralizing antibodies after at least two immunizations given four weeks apart. Interestingly, vaccination with BEI-inactivated LV also induced sizeable titers of 07V063-neutralizing antibodies. Similarly, both 07V063- and LV-based vaccines induced 08V194-neutralizing

antibodies, with the LV-induced titers being lower than the 07V063-induced titers. In all groups vaccinated with a BEI-inactivated vaccine, the VN titers dropped immediately after challenge, which may indicate that the antibodies were consumed during their interaction with virus early in infection. However, after this initial drop in VN antibody titers, VN antibodies quickly reappeared. The fast appearance of VN antibodies upon challenge is in agreement with the findings in the study of Vanhee et al. (2009) and demonstrates the potential of priming the neutralizing antibody response by immunization with a high dose of inactivated PRRSV. Although it has been reported that the PRRSV-specific neutralizing antibody response is to a large extent strain-specific and a lack in cross-neutralization may occur even between genetically closely related virus strains (Okuda et al., 2008; Kim & Yoon, 2008), our data show that cross-neutralization between genetically different isolates can occur. In the animals vaccinated with the commercial inactivated PRRSV vaccine Progressis® (first experiment), neither the IPMA nor the VN antibody response was influenced before or after challenge with 07V063. This is in line with the studies by Zuckermann et al. (2007) and Vanhee et al. (2009), where they used the same vaccine and the LV strain as challenge virus: no clear induction of challenge virus-specific (neutralizing) antibodies was observed upon vaccination with the commercial inactivated PRRSV vaccine and only a moderate anamnestic antibody response was observed upon challenge of the vaccinated animals. The apparent limited immunogenicity of this vaccine may relate to the inactivation procedure used, strain variability, antigenic dose, adjuvant, … Further research is necessary to elucidate this. In the animals vaccinated with the commercial attenuated vaccines, either based on EU- (Porcilis® PRRS) or NA- (Ingelvac® PRRS MLV) type virus, a low or non-detectable VN antibody response was observed, which is in agreement with the results of Lopez et al. (2004). None of the attenuated vaccines were able to induce a faster neutralizing antibody response upon challenge. The data obtained in this study have provided the basis for an ongoing field study on the effect of different vaccines at the farm level, more specifically on the effects of vaccination of sows on the passive immunity transferred to piglets.

In the 07V063- and 08V194-challenged groups vaccinated with a BEI-inactivated vaccine homologous to the challenge virus, a correlation was seen between the induction of virus-specific neutralizing antibodies and reduction in viremia, indicating

that VN antibodies may contribute to protection against the virus. However, the induction of homologous VN antibodies was not sufficient to completely protect the animals, as it still permitted the development of a viremia post-challenge that lasted at least one week. Possibly, higher VN antibody titers are needed at the time of challenge to offer full protection against the high dose of virus used to infect the animals. Administration of a heterologous BEI-inactivated vaccine was not sufficient to significantly reduce viremia in the animals upon challenge. Since the BEI-inactivated vaccines used in this study induced antibodies that could neutralize the homologous as well as the heterologous challenge virus in *in vitro* seroneutralization assays, it was somewhat surprising that these vaccines could only limit viremia under the homologous challenge conditions, and not when the heterologous challenge virus was used. The exact reason behind this remains currently unknown, but several possible explanations suggest themselves. For instance, it is possible that induction of virus-specific neutralizing antibodies is not sufficient and that BEI-inactivated PRRSV vaccines must promote other immune mechanisms (e.g. via cross-presentation to T-cells) to provide a significant degree of protection upon challenge. On the other hand, it can be speculated that, although the vaccine-induced antibodies can bind and neutralize the homologous and heterologous challenge virus to a similar extent in *in vitro* SN assays, they recognize the homologous virus with a higher affinity. Affinity differences may explain a reduced binding and neutralization of heterologous virus *in vivo*, as the binding conditions for (VN) antibodies are likely more stringent *in vivo* than in the *in vitro* SN assays. Under homologous challenge conditions, the antibodies have undergone optimal challenge virus-specific affinity maturation, while this is not the case under heterologous challenge conditions. In theory, the presence of vaccine-induced antibodies that cross-react with a heterologous challenge virus may even prevent the selection of high-affinity (VN) antibodies against this challenge virus (original antigenic sin). Clearly, this matter requires further investigation in the future. Despite the absence of a clear challenge virus-specific VN antibody response, the commercial attenuated vaccines do provide a partial virological protection, roughly similar to the protection provided by the autogenous BEI-inactivated vaccines. This observation points towards a significant role of other attenuated vaccine-induced immune mechanisms (e.g. cell-mediated immunity) in the protection against PRRSV infection (Charerntantanakul et al., 2006; Diaz et al., 2007; Zuckermann et al., 2007). Vaccination with the commercial

inactivated vaccine Progressis® did not induce VN antibodies and neither did it provide any degree of protection upon challenge.

Considering the similar efficacy of the attenuated vaccines against both challenge isolates used in this study, it can be questioned whether the use of autogenous inactivated vaccines is advantageous over the use of the current attenuated vaccines. However, while the efficacy of the attenuated vaccines against new virus variants can be unpredictable, our data demonstrate that an (adaptable) autogenous BEI-inactivated vaccine can provide a more or less standardized, predictable degree of protection against a specific virus variant, which may prove useful in case virus variants emerge that escape the immunity induced by the attenuated vaccines. In the near future, additional research will be conducted to further substantiate this. Also, although the production of autogenous inactivated vaccines as described in this study may appear too elaborate and costly (virus isolation, adaptation to cell culture, high dose needed,…), further optimization of the production process should make future use of these vaccines more feasible.

CONCLUSIONS

The current study assessed the protective capacity of different experimental and commercial vaccines against challenge with two recent PRRSV field isolates. Experimental BEI-inactivated vaccines based on these field isolates significantly shortened viremia upon homologous challenge. Despite the concerns regarding the efficacy of the commercial attenuated vaccines used on the farms where the field isolates were obtained, use of commercial attenuated vaccines resulted in a similar reduction of the viremic phase. In contrast, the experimental BEI-inactivated vaccines did not significantly reduce viremia upon heterologous challenge and the commercial inactivated vaccine had no apparent effect.

While the BEI-inactivated vaccines (both homologous and heterologous) induced challenge virus-specific neutralizing antibodies, this was not the case for the commercial inactivated and attenuated vaccines. The results illustrate that the capacity of a vaccine to induce challenge virus-specific neutralizing antibodies does not necessarily correlate with protection against the challenge virus and vice versa,

suggesting that not only vaccine-induced antibodies, but also other vaccine-induced immune mechanisms can contribute to PRRSV-specific protective immunity.

The observation that homologous BEI-inactivated vaccines can provide a more or less standardized, predictable degree of protection against a specific virus variant suggests that such vaccines may prove useful in case virus variants emerge that escape the immunity induced by the attenuated vaccines. Future research will allow optimization and simplification of the production process of the adaptable BEI-inactivated vaccines and give further insights into the mechanisms of protection induced by these vaccines.

ACKNOWLEDGEMENTS

The authors would like to thank Chantal Vanmaercke, Carine Boone, Chris Bracke, Nele Dennequin, Melanie Bauwens, Geert Opsomer and Bart Ellebaut for their highly appreciated and excellent technical assistance. The authors are grateful to Dominiek Maes and Tamara Vandersmissen for searching and providing farms with PRRSV-related disorders. This work was funded by the Federal Public Service Health, Food chain safety and Environment (RT - Theme 7 – Acron. PORRS) and the European Union (Seventh Framework Programme; Project No. 245141).

REFERENCES

Allende, R., Lewis, T. L., Lu, Z., Rock, D. L., Kutish, G. F., Ali, A., Doster, A. R. & Osorio, F. A. (1999). North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. *J Gen Virol* **80**, 307-315.

Cano, J. P., Dee, S. A., Murtaugh, M. P. & Pijoan, C. (2007). Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. *Vaccine* **25**, 4382-4391.

Charerntantanakul, W., Platt, R., Johnson, W., Roof, M., Vaughn, E. & Roth, J. A. (2006a). Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol* **109**, 99-115.

Christianson, W. T., Choi, C. S., Collins, J. E., Molitor, T. W., Morrison, R. B. & Joo, H. S. (1993). Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. *Can J Vet Res* **57**, 262-268.

Christianson, W. T., Collins, J. E., Benfield, D. A., Harris, L., Gorcyca, D. E., Chladek, D. W., Morrison, R. B. & Joo, H. S. (1992). Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. *Am J Vet Res* **53**, 485-488.

Delrue, I., Van Gorp, H., Van Doorsselaere, J., Delputte, P. L. & Nauwynck, H. J. (2010). Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol* **10**, 48.

Delrue, I. (2010). Development of a new inactivated porcine reproductive and respiratory syndrome virus (PRRSV) vaccine. *PhD thesis*. Ghent University, Department of Virology, Parasitology and Immunology.

Delrue, I., Delputte, P. L. & Nauwynck, H. J. (2009). Assessing the functionality of viral entry-associated domains of porcine reproductive and respiratory syndrome virus during inactivation procedures, a potential tool to optimize inactivated vaccines. *Vet Res* **40**, 62.

Dewey, C. E., Wilson, S., Buck, P. & Leyenaar, J. K., (1999). The reproductive performance of sows after PRRS vaccination depends on stage of gestation. *Prev Vet Med* **40**, 233-241.

Díaz, I., Gimeno, M., Darwich, L., Navarro, N., Kuzemtseva, L., López, S., Galindo, I., Segalés, J., Martín, M., Pujols, J. & Mateu, E. (2012). Characterization of homologous and heterologous adaptive immune responses in porcine reproductive and respiratory syndrome virus infection. *Vet Res* **43**, 30.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. (2006). Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. *Virology* **351**, 249-259.

Forsberg, R., Storgaard, T., Nielsen, H. S., Oleksiewicz, M. B., Cordioli, P., Sala, G., Hein, J. & Bøtner, A. (2002). The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. *Virology* **299**, 38-47.

Karniychuk, U. U., Saha, D., Vanhee, M., Geldhof, M. F., Cornillie, P., Caij, A. B., De Regge, N. & Nauwynck, H. J. (2012). Impact of a novel inactivated PRRSV vaccine on virus replication and virus-induced pathology in fetal implantation sites and foetuses upon challenge. *Theriogenology* **78**, 1527-1537.

Karniychuk, U. U., Saha, D., Geldhof, M., Vanhee, M., Cornillie, P., Van den Broeck, W. & Nauwynck, H. J. (2011). Porcine reproductive and respiratory syndrome virus (PRRSV) causes apoptosis during its replication in fetal implantation sites. *Microb Pathog* **51**, 194-202.

Kim, W. I. & Yoon, K. J. (2008). Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. *Virus Genes* **37**, 380-391.

Labarque, G., Reeth, K. V., Nauwynck, H., Drexler, C., Van Gucht, S. & Pensaert, M. (2004). Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. *Vaccine* **22**, 4183-4190.

Labarque, G. G., Nauwynck, H. J., Van Reeth, K. & Pensaert, M. B. (2000). Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. *J Gen Virol* **81**, 1327-1334.

Lager, K. M. & Mengeling, W. L. (1995). Pathogenesis of in utero infection in porcine fetuses with porcine reproductive and respiratory syndrome virus. *Can J Vet Res* **59**, 187-192.

Lopez, O. J., Oliveira, M. F., Garcia, E. A., Kwon, B. J., Doster, A. & Osorio, F. A. (2007). Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. *Clin Vaccine Immunol* **14**, 269-275.

Lopez, O. J. & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol* **102**, 155-163.

Martinez-Lobo, F. J., Diez-Fuertes, F., Simarro, I., Castro, J. M., & Prieto, C. (2011). Porcine reproductive and respiratory syndrome virus isolates differ in their susceptibility to neutralization. *Vaccine* **29**, 6928-6940.

Mateu, E., Martín, M. & Vidal, D. (2003). Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. *J Gen Virol* **84**, 529-534.

Meng, X. J. (2000). Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. *Vet Microbiol* **12**, 309-329.

Mengeling, W. L., Vorwald, A. C., Lager, K. M., Clouser, D.F. & Wesley, R. D. (1999). Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **60**, 334-340.

Mengeling, W. L., Lager, K. M. & Vorwald, A. C. (1994). Temporal characterization of transplacental infection of porcine fetuses with porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **55**, 1391-1398.

Nelsen, C. J., Murtaugh, M. P. & Faaberg, K. S. (1999). Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol* **73**, 270-280.

Neumann, E. J., Kliebenstein, J. B., Johnson, C. D., Mabry, J. W., Bush, E. J., Seitzinger, A. H., Green, A. L. & Zimmerman, J. J. (2005). Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. *J Am Vet Med Assoc* **227**, 385-392.

Nielsen, H. S., Oleksiewicz, M. B., Forsberg, R., Stadejek, T., Bøtner, A. & Storgaard, T. (2001). Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J Gen Virol* **82**, 1263-1272.

Nielsen, T. L., Nielsen, J., Have, P., Baekbo, P., Hoff-Jorgensen, R. & Bøtner, A. (1997). Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **54**, 101-112.

Nilubol, D., Platt, K. B., Halbur, P. G., Torremorell, M. & Harris, D. L. (2004). The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. *Vet Microbiol* **102**, 11-18.

Okuda, Y., Kuroda, M., Ono, M., Chikata, S. & Shibata, I. (2008). Efficacy of vaccination with porcine reproductive and respiratory syndrome virus following challenges with field isolates in Japan. *J Vet Med Sci* **70**, 1017-1025.

Opriessnig, T., Baker, R. B. & Halbur, P. G. (2007). Use of an experimental model to test the efficacy of planned exposure to live porcine reproductive and respiratory syndrome virus. *Clin Vaccine Immunol* **14**, 1572-1577.

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F. & Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology* **302**, 9-20.

Reed, L.J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493-497.

Rossow, K. D., Bautista, E. M., Goyal, S. M., Molitor, T. W., Murtaugh, M. P., Morrison, R. B., Benfield, D. A. & Collins, J. E. (1994). Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. *J Vet Diagn Invest* **6**, 3-12.

Scortti, M., Prieto, C., Alvarez, E., Simarro, I. & Castro, J. M. (2007). Failure of an inactivated vaccine against porcine reproductive and respiratory syndrome to protect gilts against a heterologous challenge with PRRSV. *Vet Rec* **161**, 809-813.

Scortti, M., Prieto, C., Simarro, I. & Castro, J. M. (2006b). Reproductive performance of gilts following vaccination and subsequent heterologous challenge with European strains of porcine reproductive and respiratory syndrome virus. *Theriogenology* **66**, 1884-1893.

Terpstra, C., Wensvoort, G. & Pol, J. M. (1991). Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet Q* **13**, 131-136.

Thanawongnuwech, R. & Suradhat, S. (2010). Taming PRRSV: Revisiting the control strategies and vaccine design. *Virus Res* **154**, 133-140.

Van Breedam, W., Costers, S., Vanhee, M., Geldhof, M. F., Verbeeck, M., Rodriguez Gomez, I., Van Doorsselaere, J., Gagnon, C. A. & Nauwynck, H. (2011). PRRSV-specific mAbs: supporting diagnostics and providing new insights into the antigenic properties of the virus. *Vet Immunol Immunopathol* **141**, 246-257.

Van Gucht, S., Labarque, G. & Van Reeth, K. (2004). The combination of PRRS virus and bacterial endotoxin as a model for multifactorial respiratory disease in pigs. *Vet Immunol Immunopathol* **102**, 165-178.

Vanhee, M., Delputte, P. L., Delrue, I., Geldhof, M. F. & Nauwynck, H. J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Vet. Res* **40**, 63.

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. & et al. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q* **13**, 121-130.

Zuckermann, F. A., Garcia, E. A., Luque, I. D., Christopher-Hennings, J., Doster, A., Brito, M. & Osorio, F. (2007). Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol* **123**, 69-85.

3.2

ANTIBODY RESPONSE AND MATERNAL IMMUNITY UPON BOOSTING PRRSV-IMMUNE SOWS WITH EXPERIMENTAL FARM-SPECIFIC AND COMMERCIAL PRRSV VACCINES

Marc F. Geldhof, Wander Van Breedam, Ellen De Jong, Alfonso Lopez Rodriguez, Uladzimir U. Karniychuk, Merijn Vanhee, Jan Van Doorsselaere, Dominiek Maes & Hans J. Nauwynck

Veterinary Microbiology (2013), Accepted

The porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive failure in sows and respiratory disease in pigs of all ages. Despite the frequent use of vaccines to maintain PRRSV immunity in sows, little is known on how the currently used vaccines affect the immunity against currently circulating and genetically divergent PRRSV variants in PRRSV-immune sows, i.e. sows that have a pre-existing PRRSV-specific immunity due to previous infection with or vaccination against the virus. Therefore, this study aimed to assess the capacity of commercially available attenuated/inactivated PRRSV vaccines and autogenous inactivated PRRSV vaccines – prepared according to a previously optimized in-house protocol – to boost the antibody immunity against currently circulating PRRSV variants in PRRSV-immune sows.

PRRSV isolates were obtained from 3 different swine herds experiencing PRRSV-related problems, despite regular vaccination of gilts and sows against the virus. In a first part of the study, the PRRSV-specific antibody response upon booster vaccination with commercial PRRSV vaccines and inactivated farm-specific PRRSV vaccines was evaluated in PRRSV-immune, non-pregnant replacement sows from the 3 herds. A boost in virus-neutralizing antibodies against the farm-specific isolate was observed in all sow groups vaccinated with the corresponding farm-specific inactivated vaccines. Use of the commercial attenuated EU type vaccine boosted neutralizing antibodies against the farm-specific isolate in sows derived from 2 farms, while use of the commercial

attenuated NA type vaccine did not boost farm-specific virus-neutralizing antibodies in any of the sow groups. Interestingly, the commercial inactivated EU type vaccine boosted farm-specific virus-neutralizing antibodies in sows from 1 farm. In the second part of the study, a field trial was performed at one of the farms to evaluate the booster effect of an inactivated farm-specific vaccine and a commercial attenuated EU-type vaccine in immune sows at 60 days of gestation. The impact of this vaccination on maternal immunity and on the PRRSV infection pattern in piglets during their first weeks of life was evaluated. Upon vaccination with the farm-specific inactivated vaccine, a significant increase in farm-specific virus-neutralizing antibodies was detected in all sows. Virus-neutralizing antibodies were also transferred to the piglets via colostrum and were detectable in the serum of these animals until 5 weeks after parturition. In contrast, not all sows vaccinated with the commercial attenuated vaccine showed an increase in farm-specific virus-neutralizing antibodies and the piglets of this group generally had lower virus-neutralizing antibody titers. Interestingly, the number of viremic animals (i.e. animals that have infectious virus in their bloodstream) was significantly lower among piglets of both vaccinated groups than among piglets of mock-vaccinated sows and this at least until 9 weeks after parturition.

The results of this study indicate that inactivated farm-specific PRRSV vaccines and commercial attenuated vaccines can be useful tools to boost PRRSV-specific (humoral) immunity in sows and reduce viremia in weaned piglets.

INTRODUCTION

Control of porcine reproductive and respiratory syndrome (PRRS) is a major challenge to all people involved in the swine industry. The causative agent of PRRS, the PRRS virus (PRRSV), is a positive-sense single-stranded RNA virus that belongs to the family of the *Arteriviridae*, order *Nidovirales* (Meng, 2000). PRRSV infections are associated with respiratory distress in swine of all ages and reproductive failure in the breeding stock. Clinical signs reported in PRRSV-infected gilts and sows include late-term abortion (days 107 – 110 of gestation), early farrowing (days 110 – 112 of gestation) and high numbers of stillborn, mummified or weakborn piglets (Collins et al., 1992; Wensvoort et al., 1991). Losses in the breeding and farrowing, the nursery and the grower-finisher phase due to PRRSV impose a substantial economic burden on pork producers (Neumann et al., 2005). Historically, PRRSV isolates are divided into two genotypes: a European (EU, type I) and a North American (NA, type II) genotype. The genetic, antigenic and pathogenic variability (Bautista et al., 1993; Halbur et al., 1996; Meng et al., 1995; Nelson et al., 1993) within each genotype is high and is increasing in time. This genetic drift presents a real challenge for PRRSV control at herd and regional level (Meng, 2000).

The most commonly used strategy to combat PRRSV is to force back the virus through vaccination. Commercial attenuated and inactivated vaccines, either based on EU or NA type PRRSV, are routinely used in gilts, sows and growing pigs in order to reduce the negative effects of PRRSV infection. Commercial attenuated PRRSV vaccines offer clear protection against (re-) infection with homologous virus variants (Labarque et al., 2004; Martelli et al., 2007; Nielsen et al., 1997; Plana-Duran et al., 1997; Zuckermann et al., 2007) and the use of these vaccines in PRRSV-affected populations was reported to reduce the number of persistently infected and shedding pigs (Cano et al., 2007). Unfortunately, they seem to protect less completely and inconsistently against heterologous viruses (Geldhof et al., 2012; Kimman et al., 2009; Labarque et al., 2004; Murtaugh et al., 2002). In addition, there have been some safety issues with the current generation of commercial attenuated vaccines. It has been described that the vaccine virus may spread transplacentally and/or cause reproductive failure in sows and gilts (Nielsen et al., 2001). Moreover, the vaccine virus may revert to virulence and cause pathology (Mengeling et al., 1999; Nielsen et al., 2001; Nielsen et al., 2002) and there is even evidence of recombination between

attenuated vaccine virus and circulating PRRSV variants in the field (Li et al., 2009). In contrast with the attenuated vaccines, the commercial inactivated vaccines are generally safe. A significant drawback however is that they appear to have limited efficacy. While some studies suggest a correlation between the use of these vaccines and an improved farrowing rate, reduction in return to oestrus and an increase in the number of piglets weaned per sow in endemically infected populations (Papatsiros et al., 2006), several other studies report that commercial inactivated vaccines do not protect gilts against an experimental PRRSV challenge (Scortti et al., 2007; Zuckermann et al., 2007). Interestingly however, it was recently demonstrated that an inactivated PRRSV vaccine can be developed that offers partial protection against experimental challenge after 2 vaccinations (Vanhee et al., 2009). Moreover, it was shown that this vaccine can be easily adapted to farm-specific PRRSV variants (Geldhof et al., 2012), which is interesting since virus heterogeneity can compromise vaccine efficacy and since the degree of protection induced by vaccination is linked to the homology between the vaccine virus and the field virus to which the pigs are exposed (Labarque et al., 2004).

Different immune mechanisms seem to be involved in protection against PRRSV and there is ample evidence that the PRRSV-specific antibody response may play a significant role in this process. It has for instance been shown that virus-neutralizing antibodies can protect pregnant sows against PRRSV-associated reproductive failure and transplacental spread, as well as against virus replication in tissues and viremia upon challenge with infectious virus (Lopez and Osorio 2004; Osorio et al., 2002). In addition, passive transfer of sufficient amounts of virus-specific neutralizing antibodies can prevent viremia in young weaned pigs (Lopez et al., 2007). Conceivably, virus-specific (neutralizing) antibodies are also of major importance for maternal immunity: it is well known that the colostrum is the primary source of protective antibodies and other (pathogen-specific) immune factors in newborn piglets (Nechvatalova et al., 2011). The current generation of commercial attenuated vaccines can induce antibodies that neutralize homologous virus variants (Charerntantanakul et al., 2006; Okuda et al., 2008; Scortti et al., 2006). Similarly, the recently developed experimental inactivated PRRSV vaccine has the capacity to induce neutralizing antibodies in naïve animals (Geldhof et al., 2012; Vanhee et al., 2009). This is in contrast with the commercially available inactivated vaccines, which are not able to

induce a robust (neutralizing) antibody response (Geldhof et al., 2012; Vanhee et al., 2009; Zuckermann et al., 2007). Despite the knowledge on their antibody-inducing capacity and their frequent use to control the disease in breeding herds, little is known on how the commercial attenuated and inactivated vaccines may boost the (neutralizing) antibody response against currently circulating PRRSV variants in PRRSV-immune sows, i.e. sows that have a pre-existing PRRSV-specific immunity due to previous infection with or vaccination against the virus. It is also not known to what extent the recently developed farm-specific inactivated PRRSV vaccines may boost antibody immunity against newly emerging virus variants in PRRSV-immune animals.

The present study aimed to evaluate the antibody immunity against currently circulating PRRSV variants in PRRSV-immune sows upon booster vaccination with farm-specific inactivated PRRSV vaccines and commercial PRRSV vaccines. A first experiment examined the effect of booster vaccination with these vaccines on the antibody response in non-pregnant, PRRSV-immune replacement sows under experimental conditions. Upon vaccination, virus-specific as well as virus-neutralizing antibody concentrations in the blood were monitored and animals were checked for viremia. A second experiment focussed on the antibody response in pregnant, PRRSV-immune sows after booster vaccination under field conditions. This experiment was performed on a farm where PRRSV is endemic, despite regular vaccination against the virus. Virus-specific as well as virus-neutralizing antibody concentrations in the blood of the sows were monitored and animals were checked for viremia. After parturition and transfer of colostrum to the piglets, the same parameters were also monitored in their offspring.

MATERIALS AND METHODS

Virus isolates

PRRSV isolates were obtained from 3 farms, showing clinical signs compatible with PRRS in sows and growing pigs despite vaccination against PRRSV. Isolate 07V063 was isolated from fetal tissue, while PRRSV isolates 08V194 and 08V204 were isolated from the serum of a 14- and a 4-week-old piglet, respectively. All 3 isolates were isolated and cultured on porcine alveolar macrophages (PAM) and subsequently

adapted to growth on MARC-145 cells by repeated passages. Sequencing of ORF2-7 of these isolates was performed as described before (Delrue et al., 2010; Geldhof et al., 2012). Nucleotide sequences were submitted to Genbank under accession numbers: [GU737264], 07V063; [GU737265], 08V194; and [GU737266], 08V204. Sequencing data allowed their classification as EU type viruses and pointed out they did not originate from attenuated vaccine viruses used in the farms (Farms 07V063 and 08V204: Porcilis® PRRS, Intervet at 60 days of pregnancy and Ingelvac® PRRS MLV, Boehringer Ingelheim at 6 days of lactation; Farm 08V194: Ingelvac® PRRS MLV, Boehringer Ingelheim every 4 months). MARC-145 grown stocks of PRRSV 07V063 (2 passages on PAM + 2 passages on MARC-145), 08V194 (2 passages on PAM + 4 passages on MARC-145) and 08V204 (2 passages on PAM + 3 passages on MARC-145) were prepared for experimental vaccine preparation.

Vaccines

Binary ethyleneimine (BEI) -inactivated vaccines, based on the farm-specific PRRSV isolates 07V063, 08V194 and 08V204, were prepared (07V063i, 08V194i and 08V204i). Vaccine preparation and quality control were performed as described before (Geldhof et al., 2012; Vanhee et al., 2009). Each vaccine dose consisted of 1 mL BEI-inactivated, MARC-145-grown virus (10^8 $TCID_{50}$ on MARC-145 cells) mixed with 1 mL o/w Suvaxyn (an oil-in-water diluent, normally used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky, Fort Dodge Animal Health). BEI-inactivated vaccines were administered intramuscularly in the neck muscles behind the ear.

The commercial PRRSV vaccines used in this study include an attenuated EU type vaccine (Porcilis® PRRS, Intervet, ≥ 10^4 $TCID_{50}$/ 2 mL), an attenuated NA type vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim, ≥ $10^{4,9}$ $TCID_{50}$/ 2 mL) and an inactivated EU type vaccine (Progressis®, Merial, strain P120: ≥ 2,5 log IF Units).

Vaccination of non-pregnant PRRSV-immune replacement sows under experimental conditions

Animals

Twenty-three to twenty-five non-pregnant culled sows were selected from each of the 3 PRRSV-positive farms and housed in A2 biosafety level animal facilities. At that time, all 3 farms applied a vaccination scheme using commercial vaccines (farm 07V063 and farm 08V204: EU type attenuated vaccine at 60 days of pregnancy, NA type attenuated vaccine at 6 days of lactation; farm 08V194: NA type attenuated vaccine every 4 months) in sows. Weaners, growers and finishing pigs were not vaccinated. Upon arrival of the animals in the animal facilities, serum samples of all selected sows were tested and found negative in virus isolation assays on alveolar macrophages and MARC-145 cells.

At the time of selection, each farm also submitted 50 serum samples: 10 of sows, 10 of gilts, and 10 of 3-, 7-, and 10-week-old piglets. Serologic testing via immunoperoxidase monolayer assay (IPMA) revealed high titers of PRRSV-specific antibodies in animals of all age groups. Selected sera with high PRRSV-specific antibody titers were used for virus isolation on alveolar macrophages. The virus isolation assays revealed that PRRSV was circulating at the 3 farms. Sequencing of ORF7 of the virus isolates revealed that all viruses had the same background as the viruses that had been isolated earlier on the respective farm and that were used for vaccine preparation.

Experimental design

The sows from each herd were randomly assigned to five different groups (Table 1). Groups 07V063CON, 08V194CON and 08V204CON (n = 3 or 5) served as mock-vaccinated control groups, receiving 1 mL RPMI-1640 in 1 mL o/w Suvaxyn. The sows of group 07V063i, 08V194i and 08V204i (n = 5) were vaccinated with a BEI-inactivated autogenous PRRSV vaccine based on the herd-specific PRRSV isolate. Groups 07V063PROi, 08V194PROi and 08V204PROi (n = 5) received 2 mL of a commercial EU type inactivated PRRSV vaccine (Progressis®). Groups 07V063PORatt, 08V194PORatt, 08V204PORatt (n = 5) and 07V063INGatt, 08V194INGatt, 08V204INGatt (n = 5) were vaccinated with an EU- (Porcilis®

PRRS) and an NA- (Ingelvac® PRRS MLV) type attenuated vaccine, respectively. In all groups, vaccination was performed only once (single shot), one week upon arrival of the animals in the animal facilities. Following vaccination, general health, appetite and rectal body temperature of the sows were monitored on a daily basis. The injection site was examined for local inflammation. Animals were bled at 0, 1, 2 and 3 weeks post vaccination and serum was collected for detection of virus-specific and virus-neutralizing antibodies via IPMA and seroneutralization (SN) tests, respectively. Serum samples were also examined for the presence of infectious virus using virus isolation assays on PAM and MARC-145 cells. All animal experiments were approved by the local ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Vaccination of pregnant PRRSV-immune sows under field conditions

Animals / Farm

The herd used in this experiment was a 340-sow farrow-to-finish farm, located in West-Flanders (Belgium). The farm suffered from endemic PRRSV infection since a severe PRRSV-outbreak in 2007. At the start of the experiment, circulation of the PRRSV isolate 07V063 in the herd was confirmed by virus isolation and sequencing of ORF7. The owners agreed to participate in a field trial to test the antibody response in 30 pregnant, PRRSV-immune sows after vaccination with a farm-specific BEI-inactivated vaccine or an attenuated EU type PRRSV vaccine regularly used on the farm (Porcilis® PRRS, Intervet) and to evaluate the protection by colostral immunity in their offspring.

Experimental design

Thirty pregnant sows were randomly assigned to 3 different groups (Table 1). Ten sows were mock-vaccinated (1 mL RPMI-1640 in 1 mL o/w Suvaxyn) and served as a control group (group 07V063CON2). Ten sows were vaccinated with a BEI-inactivated autogenous PRRSV vaccine, based on the PRRSV variant isolated on the farm (group 07V063i2). Ten sows were vaccinated with an attenuated EU type PRRSV vaccine (Porcilis® PRRS, Intervet) (group 07V063PORatt2). All vaccines were administered once at 60 days of gestation. Following vaccination, general health, appetite and rectal body temperature of the sows were monitored on a daily

basis. Blood was taken from all sows at the time of vaccination, 2 weeks later, and at the beginning and end of the lactation period. After parturition, 4 piglets were randomly selected from each sow and blood samples were taken from these animals at fixed time-points (3, 5, 7 and 9 weeks of age). Virus-specific and virus-neutralizing antibody titers were determined via IPMA and SN tests, respectively. Serum samples were examined for the presence of infectious virus using virus isolation assays on PAM and MARC-145 cells. This animal experiment was approved by the local ethical committee of the Faculty of Veterinary Medicine, Ghent University. The FAGG and FAVV gave their permission (pharmaceutical preparation according to the cascade arrangement) for the field trial study.

Table 1: Overview of all groups in both experiments

Experiment 1: Experimental conditions				
	Group		***Number of sows***	***Vaccination (single shot; non-pregnant sows)***
07V063	***08V194***	***08V204***		
07V063CON	08V194CON	08V204CON	3 or 5	Mock vaccine
07V063i	08V194i	08V204i	5	BEI-inactivated PRRSV isolate
07V063PROi	08V194PROi	08V204PROi	5	Progressis®, Merial
07V063PORatt	08V194PORatt	08V204PORatt	5	Porcilis® PRRS, Intervet
07V063INGatt	08V194INGatt	08V204INGatt	5	Ingelvac® PRRS MLV, Boehringer Ingelheim
Experiment 2: Field conditions				
	Group		***Number of sows***	***Vaccination (single shot at 60 days of gestation)***
	07V063CON2		10	Mock vaccine
	07V063i2		10	BEI-inactivated 07V063-isolate
	07V063PORatt2		10	Porcilis® PRRS, Intervet

Serological examination and virus isolation assays

PRRSV-specific antibody titers in serum samples were determined using IPMA assays as described by Wensvoort et al. (1991). IPMA tests were performed on MARC-145 cells infected with the MARC-145-adapted PRRSV isolates 07V063, 08V194 or 08V204. For each animal group, IPMAs were performed on cells infected with the virus variant isolated on the corresponding farm. Virus-neutralizing antibodies were detected by SN tests on MARC-145 cells as described before (Geldhof et al., 2012), using the MARC-145-adapted isolates 07V063, 08V194 or 08V204. For each animal group, SN tests were performed with the virus variant isolated on the corresponding farm.

To detect viremia (i.e. the presence of infectious virus in the bloodstream), presence of infectious PRRSV in serum samples was tested using virus isolation assays as

described before (Labarque et al., 2000). To check the sensitivity of the PAM and different passages of MARC-145 cells, all used cell batches/passages were assayed in virus titrations using PRRSV stocks with known virus titers.

Statistical analysis

The serology of the sow sera was analyzed using a Friedman test, followed by Dunn's multiple comparisons test to determine significant differences compared to the day of vaccination at different time-points post vaccination in all groups.

In the second experiment, a two-tailed Fisher's exact test was used to detect significant differences in the number of piglets with maternal virus-neutralizing antibodies in the 3 piglet groups. The same test was used to determine significant differences in the number of viremic animals in the 3 piglet groups. A Kruskall-Wallis test, followed by Dunn's multiple comparisons test, was performed to detect significant differences in maternal virus-neutralizing antibody titers between the 3 piglet groups.

An overall p value of 0.05 was taken as the level of statistical significance. All statistical analyses were performed using GraphPad Prism version 5.0a (GraphPad Software, San Diego, California, USA).

RESULTS

Vaccination of non-pregnant PRRSV-immune replacement sows under experimental conditions

Clinical signs and detection of infectious PRRSV in sow serum samples

All sows remained in good health after they were vaccinated. No local or systemic vaccine side effects were noted throughout the trial period. At all time-points, the serum samples of all animals were PRRSV-negative as determined by virus isolation assays on PAM and MARC-145 cells.

Antibody response

On the day of vaccination, all animals were positive for PRRSV-specific antibodies and many also for virus-neutralizing antibodies. Upon vaccination, no significant increase in virus-specific or virus-neutralizing antibodies was seen in the mock-vaccinated groups 07V063CON, 08V194CON and 08V204CON. When compared to the day of vaccination, significantly higher virus-specific antibody titers were observed in group 07V063i at 2 and 3 weeks post vaccination (Figure 1). Upon vaccination, group 07V063i showed a significant increase in virus-neutralizing antibodies at 2 and 3 weeks post vaccination reaching mean values of 7.0 log_2 and 7.2 log_2, respectively (Figure 2). In group 08V194i, the virus-neutralizing antibody titers were significantly higher at 2 and 3 weeks post vaccination, reaching mean values of 5.2 log_2 and 5.3 log_2, respectively (Figure 2). Group 08V204i showed a significant increase in virus-neutralizing antibodies at 2 and 3 weeks post vaccination, reaching mean values of 5.7 log_2 and 4.9 log_2, respectively (Figure 2). For group 08V204PROi, virus-specific antibody titers were significantly increased at 2 and 3 weeks post vaccination (Figure 1). No differences in virus-neutralizing antibodies were observed at any time-point in groups 07V063PROi and 08V194PROi. Group 08V204PROi showed a significant increase in virus-neutralizing antibodies at 3 weeks post vaccination, reaching a mean value of 3.3 log_2 (Figure 2). Upon vaccination, virus-specific antibodies were significantly increased at 2 and 3 weeks post vaccination for group 08V204PORatt (Figure 1). A significant increase in virus-neutralizing antibodies was detected in group 08V194PORatt at 2 and 3 weeks post vaccination, reaching mean values of 2.7 log_2 and 2.8 log_2 respectively, and in group 08V204PORatt at 2 weeks post vaccination, reaching a mean value of 4.8 log_2. No increase in virus-neutralizing antibodies was detected in 07V063PORatt (Figure 2). Upon vaccination with Ingelvac® PRRS MLV, no differences in virus-specific or virus-neutralizing antibody titers were observed in the groups 07V063INGatt, 08V194INGatt and 08V204INGatt, at any time-point tested (Figure 1-2).

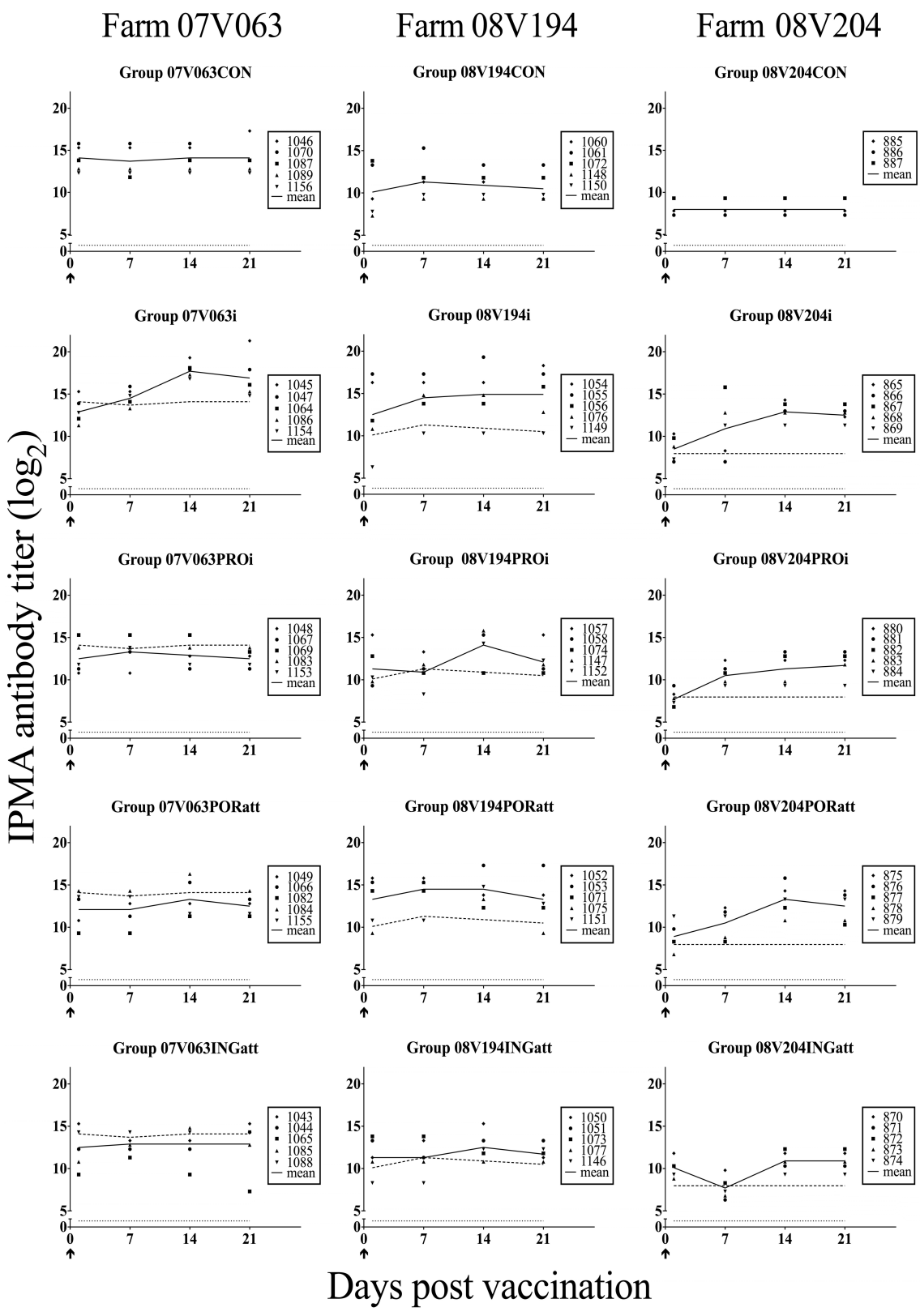


Figure 1. PRRSV-specific (IPMA) antibody titers (log_2) after vaccination in sow groups 07V063CON, 08V194CON, 08V204CON (Mock-vaccinated control), 07V063i, 08V194i, 08V204i (BEI-inactivated PRRSV isolate), 07V063PROi, 08V194PROi, 08V204PROi (Progressis®), 07V063PORatt, 08V194PORatt, 08V204PORatt (Porcilis® PRRS) and 07V063INGatt, 08V194INGatt and 08V204INGatt (Ingelvac® PRRS MLV). ↑ = Day of vaccination. Symbols represent individual animals and solid lines represent mean IPMA titers calculated on all animals in each group. The dotted line marks the detection limit for the IPMA test.

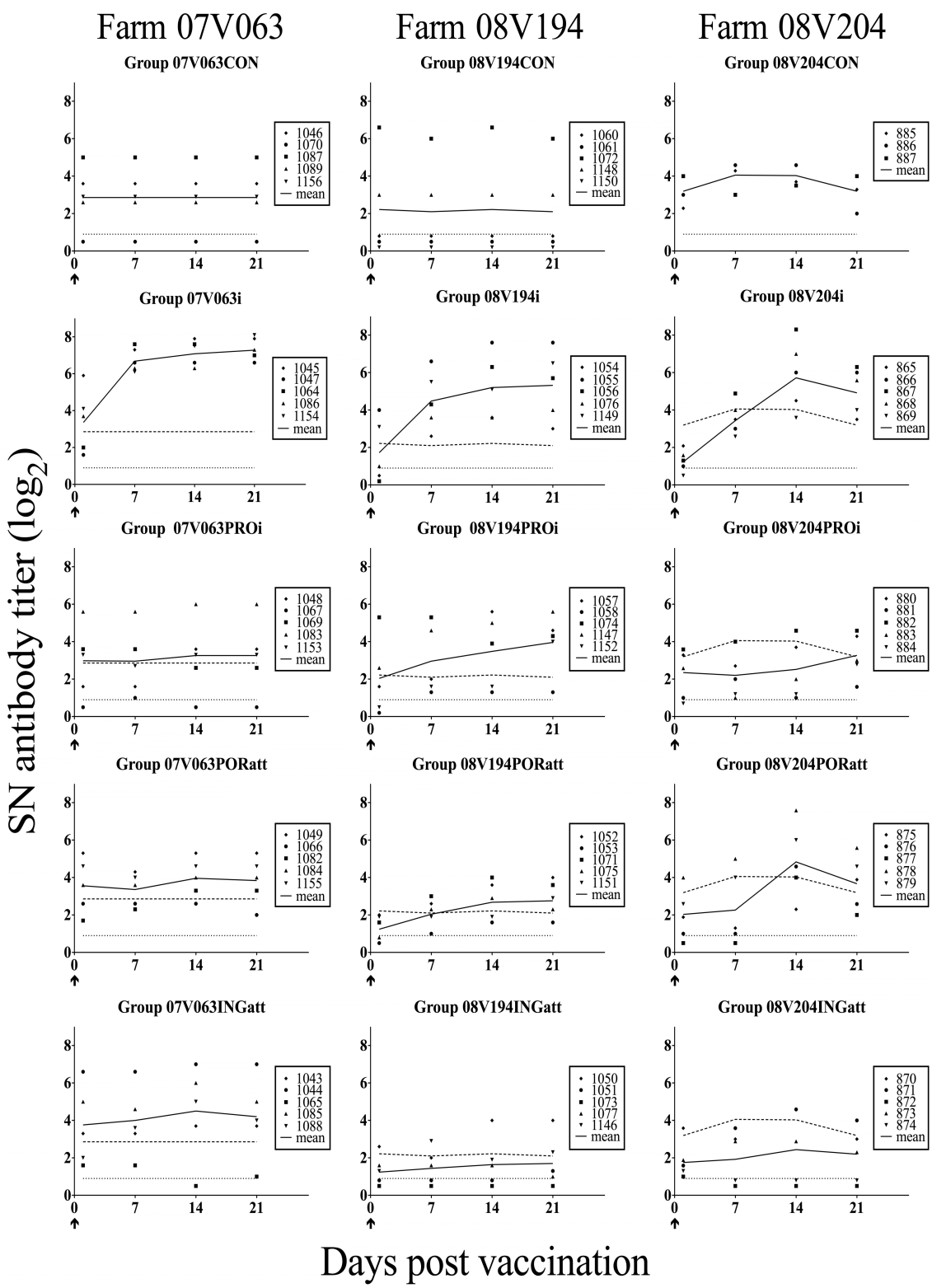


Figure 2. PRRSV-neutralizing (SN) antibody titers (log_2) after vaccination in sow groups 07V063CON, 08V194CON, 08V204CON (Mock-vaccinated control), 07V063i, 08V194i, 08V204i (BEI-inactivated PRRSV isolate), 07V063PROi, 08V194PROi, 08V204PROi (Progressis®), 07V063PORatt, 08V194PORatt, 08V204PORatt (Porcilis® PRRS) and 07V063INGatt, 08V194INGatt and 08V204INGatt (Ingelvac® PRRS MLV). ↑ = Day of vaccination. Symbols represent individual animals and solid lines represent mean SN titers calculated on all animals in each group. The dotted line marks the detection limit for the SN test.

Vaccination of pregnant PRRSV-immune sows under field conditions

Clinical signs and detection of infectious PRRSV in sow serum samples

Upon vaccination, the appetite of all sows remained normal and no local or general reactions were observed. One of the mock-vaccinated sows died during parturition. Eight piglets died during nursery and the finding of traumatic lesions in the head and/or body together with milk-filled stomachs indicated that 5 of these piglets were crushed to death by the dams. Routine diagnosis, including a virus isolation assay for PRRSV detection, was performed on tissue samples of the 3 remaining piglets, but samples were negative for all tested pathogens. No infectious PRRSV was isolated from any of the sow serum samples (either on PAM or MARC-145 cells).

Antibody response

Sows

On the day of vaccination, all animals were positive for PRRSV-specific antibodies and the majority also for virus-neutralizing antibodies. The sows of group 07V063CON2 showed no significant increase in virus-specific or virus-neutralizing antibodies at any time-point post vaccination (Figure 3-4). Upon vaccination, a significant increase in virus-specific antibody titers was observed at 2 weeks post vaccination and at the end of lactation in group 07V063i2 (Figure 3) and a significant increase in virus-neutralizing antibody titers was observed at 2 weeks post vaccination, reaching a mean titer of 5.6 $\log_2$ (Figure 4). Upon vaccination with Porcilis® PRRS, no significant increase in virus-specific or virus-neutralizing antibody titers was observed at any time-point.

Piglets

Virus-specific antibodies were found in serum samples taken from piglets born to all sows. At 3 weeks of age, the virus-specific antibody titers of piglets from sow group 07V063i2 were significantly higher than those of piglets of sow group 07V063CON2, but this was not the case for the 07V063PORatt2-derived piglets. While the virus-specific antibody titers in the majority of piglets from sow groups 07V063i2 and 07V063PORatt2 decreased or remained stable over time, the virus-specific antibody titers in most piglets derived from the 07V063CON2 sow group increased towards the

end of the trial (data not shown). Four piglets derived from 3 sows of group 07V063CON2 had a low virus-neutralizing antibody titer at 3 weeks of age and only 2 piglets from 1 sow of group 07V063CON2 had virus-neutralizing antibody titers at 5 weeks of age.

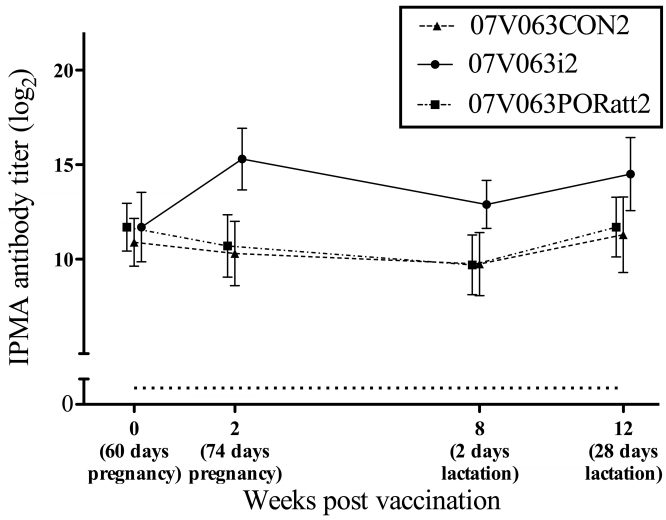


Figure 3. PRRSV-specific (IPMA) antibody titers (log_2) after vaccination for group 07V063CON2 (Mock-vaccinated control), 07V063i2 (BEI-inactivated 07V063) and 07V063PORatt2 (Porcilis® PRRS) sows. Symbols represent mean IPMA titers (± standard deviation) calculated on all animals in each group. The dotted line marks the detection limit for the IPMA test.

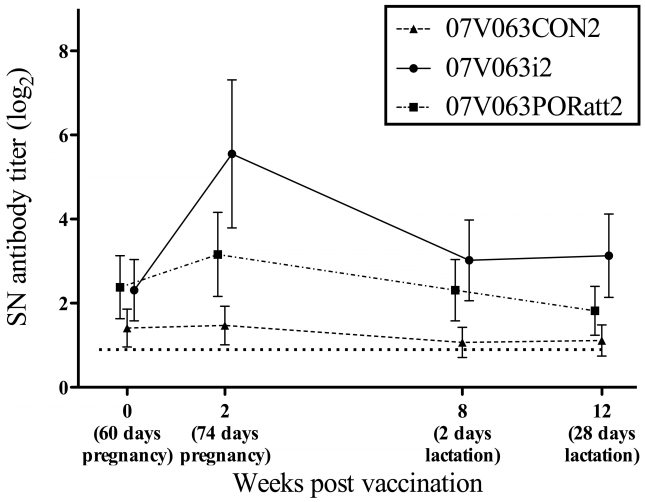


Figure 4. PRRSV-neutralizing (SN) antibody titers (log_2) after vaccination for group 07V063CON2 (Mock-vaccinated control), 07V063i2 (BEI-inactivated 07V063) and 07V063PORatt2 (Porcilis® PRRS) sows. Symbols represent mean SN titers (± standard deviation) calculated on all animals in each group. The dotted line marks the detection limit for the SN test.

No virus-neutralizing antibodies against 07V063 were detected in any of these piglets at other time-points. At 3 weeks of age, 100% (37/37) and 66% (25/38) of piglets from sow groups 07V063i2 and 07V063PORatt2 had virus-neutralizing antibody titers, respectively. Virus-neutralizing antibody titers had disappeared in a majority of the piglets of sow group 07V063PORatt2 (29% positive, 11/38) by 5 weeks of age, while 81% (30/37) of piglets of sow group 07V063i2 were still positive at that time. At 7 and 9 weeks of age, 2 piglets of 1 sow of group 07V063i2 still had virus-neutralizing antibodies against 07V063. At these time-points, no virus-neutralizing antibodies were detected in the serum of piglets derived from any other sow. When compared with group 07V063CON2 and 07V063PORatt2 piglets, a significantly higher number of animals with virus-neutralizing antibodies was observed in group 07V063i2 piglets at 3 and 5 weeks of age. Also, the virus-neutralizing antibody titers in group 07V063i2 piglets were significantly higher at 3 and 5 weeks compared to group 07V063CON2 and 07V063PORatt2 piglets (Figure 5).

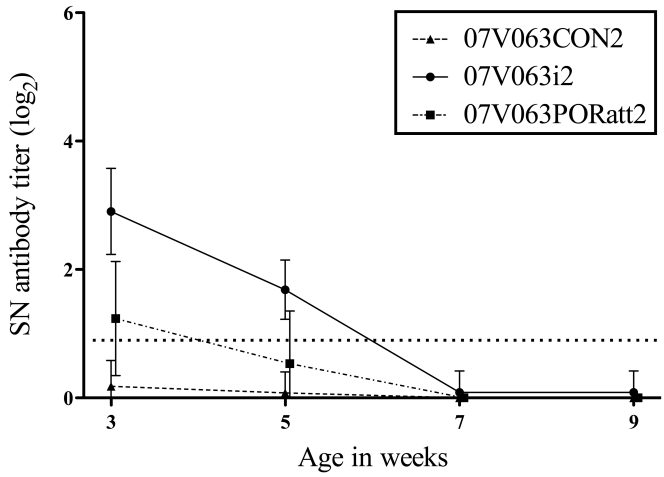


Figure 5. PRRSV-neutralizing (SN) antibody titers (log_2) for the piglets derived from group 07V063CON2 (Mock-vaccinated control), 07V063i2 (BEI-inactivated 07V063) and 07V063PORatt2 (Porcilis® PRRS) sows. Symbols represent mean SN titers (± standard deviation) calculated on all piglets in each sow group. The dotted line marks the detection limit for the SN test.

Detection of infectious PRRSV in piglet serum samples

The results of virus isolation on PAM cells from piglet serum samples are shown in Table 2. Infectious PRRSV was isolated from 2 serum samples of 3-week-old piglets of 1 sow of group 07V063CON2, resulting in a PRRSV-positive percentage of 6% in

this group. At that time, no infectious virus could be isolated from any of the piglets of group 07V063i2 and 07V063PORatt2 sows. In the 07V063i2 and 07V063PORatt2 piglet groups, the first viremic animals were only observed at 7 weeks of age. At 9 weeks, infectious PRRSV was isolated from 64%, 21% and 19% of piglets from group 07V063CON2, 07V063PORatt2 and 07V063i2 sows, respectively. When compared to group 07V063CON2 piglets, the number of viremic animals in group 07V063i2 and group 07V063PORatt2 piglets was significantly lower at 7 and 9 weeks of age.

Table 2: Viremia in piglets per sow per group

Group	Sow number	Number of piglets	Number of viremic piglets at … weeks of age			
			3	5	7	9
07V063CON2	1	4	0	0	3	3
	2	2	0	0	0	0
	3	**				
	4	4	0	0	3	3
	5	4	0	0	0	1
	6	3	0	0	1	2
	7	4	2	0	2	3
	8	4	0	0	2	3
	9	4	0	1	2	3
	10	4	0	0	0	3
07V063i2	11	4	0	0	0	0
	12	4	0	0	0	0
	13	4	0	0	0	0
	14	4	0	0	0	1
	15	3	0	0	0	0
	16	3	0	0	0	1
	17	4	0	0	1	2
	18	4	0	0	0	2
	19	3	0	0	1	1
	20	4	0	0	0	0
07V063PORatt2	21	3	0	0	0	1
	22	4	0	0	0	0
	23	4	0	0	0	0
	24	3	0	0	0	1
	25	4	0	0	0	1
	26	4	0	0	0	0
	27	4	0	0	0	0
	28	4	0	0	0	0
	29	4	0	0	0	2
	30	4	0	0	1	3

** = Sow died during parturition.

DISCUSSION

PRRSV is an economically important pathogen of pigs causing respiratory distress in piglets and reproductive failure in sows. PRRSV outbreaks on breeding farms manifest themselves in the form of late abortions, premature farrowing or birth of dead and/or weak piglets. Vaccination of sows is one of the strategies used to minimize the clinical and economic impact of PRRSV infections. However, the currently used vaccines appear to have variable degrees of success. In the last years, many pig farms routinely vaccinating their sows with the commercially available vaccines have suffered infections with new virus variants that escape the immunity induced by the current vaccine strains (Thanawongnuwech & Suradhat, 2010). The high genetic and antigenic variability of PRRSV clearly poses an important challenge for herd-level and regional control (Murtaugh et al., 1995; Kapur et al., 1996; Nelsen et al., 1999; Kimman et al., 2009;). Although all the immune factors that can contribute to an effective protection and virus clearance have not yet been identified, several studies point out an important role of virus-neutralizing antibodies. Passive transfer of PRRSV-specific virus-neutralizing antibodies can protect pregnant sows against reproductive failure and confer sterilizing immunity in sows and offspring (Osorio et al., 2002). Similarly, passive transfer of sufficient amounts of virus-specific neutralizing antibodies can prevent viremia in young weaned pigs (Lopez et al., 2007). In addition, virus-specific neutralizing antibodies may significantly contribute to the colostral immunity that protects suckling piglets during their first weeks of life (Nechvatalova et al., 2011). Despite the frequent use of commercially available attenuated and inactivated vaccines for maintaining immunity in breeding herds, little is known on how these vaccines boost the antibody immunity against divergent (wild-type) virus variants in PRRSV-immune animals. This study aimed to evaluate the antibody immunity against currently circulating PRRSV variants in PRRSV-immune sows upon booster vaccination with farm-specific inactivated PRRSV vaccines and commercial PRRSV vaccines.

A first experiment was performed to evaluate the serological response of non-pregnant replacement sows, originating from herds with active circulation of naturally occurring PRRSV-variants, upon vaccination with a farm-specific BEI-inactivated

vaccine or either of 3 commonly used commercial vaccines (inactivated or attenuated). Upon vaccination, sows that received a farm-specific inactivated vaccine showed a significant increase in serum virus-neutralizing antibodies specific for the prevalent PRRSV-variant in their herd. Previous studies in piglets have shown that this type of vaccine can induce virus-specific neutralizing antibodies in naïve animals (Vanhee et al., 2009; Geldhof et al., 2012). In contrast, vaccination with the commercial inactivated vaccine gave variable results: the vaccine boosted the 08V204-specific, but not the 07V063- or 08V194-specific virus-neutralizing antibody response. Although it has been previously reported that these vaccines may boost virus-neutralizing antibody production in previously infected animals (Nilubol et al., 2004 Zimmerman et al., 2006), several studies suggest that the commercial inactivated vaccines do not significantly stimulate the virus-neutralizing antibody response in naïve animals (Zuckermann et al., 2007; Vanhee et al., 2009; Geldhof et al., 2012). Although substantial experimental proof is currently lacking, the apparent limited immunogenicity of the commercial inactivated vaccines may be related to the inactivation procedure, strain variability, antigenic dose, adjuvant, … Also vaccination with the commercial attenuated vaccines gave variable results. Vaccination with the attenuated EU type vaccine (Porcilis® PRRS) increased 08V194- and 08V204-specific virus-neutralizing antibody titers in sows derived from the respective farms, but did not boost the 07V063-specific virus-neutralizing antibody titers in sows of farm 07V063. Vaccination with the attenuated NA type vaccine (Ingelvac® PRRS MLV) did not boost the virus-neutralizing antibody production against any of the farm-specific isolates. These results support the idea that current generation attenuated vaccines are not universally successful in stimulating the (heterologous) humoral immune response in PRRSV-immune animals. Although the absence of an anamnestic humoral immune response upon infection or re-vaccination may result from a lack in sufficient vaccine virus replication in PRRSV-immune animals, it is conceivable that antigenic differences between the vaccine and challenge virus contribute significantly to this phenomenon.

A second experiment investigated the impact of a BEI-inactivated autogenous vaccine and a commercial attenuated vaccine administered at 60 days of gestation on maternal immunity and on the PRRSV infection pattern in piglets during their first weeks of

life.

In sows vaccinated with the farm-specific BEI-inactivated vaccine, 07V063-specific virus-neutralizing antibodies in the serum were significantly increased at 2 weeks post vaccination. In contrast, vaccination with Porcilis® PRRS did not significantly increase 07V063-specific virus-neutralizing antibody titers in the sow sera at any time-point post vaccination. At 3 and 5 weeks postpartum, a significantly higher number of animals with 07V063-specific virus-neutralizing antibodies was observed in group 07V063i2 piglets compared to group 07V063CON2 and 07V063PORatt2 piglets. Also, the 07V063-specific virus-neutralizing antibody titers in group 07V063i2 piglets were significantly higher at 3 and 5 weeks compared to group 07V063CON2 and 07V063PORatt2 piglets.

The appearance of viremic animals in the 07V063i2- and 07V063PORatt2-derived piglet groups was delayed in time and the number of piglets that became viremic within the trial period was significantly reduced compared with the 07V063CON2-derived piglet group. Conceivably, the passive transfer of 07V063-specific virus-neutralizing antibodies in colostrum of 07V063i2-vaccinated sows to piglets contributed to the curtailment in the number of viremic piglets. In line with this, previous studies have shown that passive transfer of virus-neutralizing antibodies can protect young piglets against PRRSV viremia (Lopez et al., 2007). Despite a lower transfer of virus-neutralizing antibodies from sow to piglets in Porcilis® PRRS-vaccinated sows, results from our study indicate that the use of such a vaccine in sows still has positive effects in the nursery. These data suggest that also other colostrum-derived immune factors may play a role in the immunity of these piglets. As previously documented, not only antibodies, but also other immune factors/components (cytokines, immune cells, antibacterial proteins, lysozymes, …) can be transferred via colostrum (Nguyen et al., 2007; Bandrick et al., 2008; Salmon et al., 2008; Nechvatalova et al., 2011). Data obtained in the current study illustrate the potential of maternal vaccination to protect piglets from PRRSV-infection during their first weeks of life. Further research will yield a better understanding of protective PRRSV-specific maternal immunity.

CONCLUSION

None of the currently available PRRSV vaccines is able to completely prevent respiratory infection, transplacental transmission and pig-to-pig transmission of wild-type virus variants or maintain immune protection in sows (Murtaugh et al., 2002). In spite of this, producers should not rule out using PRRSV vaccines as an aid to control clinical PRRSV. Vaccines have been used successfully to reduce the negative effects of PRRSV infection (Murtaugh et al., 2002; Kimman et al., 2009). In line with this, the results of the current study indicate that attenuated and inactivated vaccines can be useful tools to boost PRRSV-specific (humoral) immunity in PRRSV-immune sows and reduce viremia in weaned piglets. Farm-specific inactivated vaccines may prove useful in vaccination programs to boost the immunity in pregnant sows. Future research will allow optimization and simplification of the production process of the adaptable BEI-inactivated vaccines and give further insights into the mechanisms of protection induced by these vaccines.

ACKNOWLEDGEMENTS

The authors would like to thank Chantal Vanmaercke, Carine Boone, Melanie Bauwens, Nele Dennequin, Geert Opsomer, Bart Ellebaut, Zeger Vandenabeele, Annelies Sierens, Ruben Del Pozo and Ruben Decaluwé for excellent technical assistance.

This work was funded by the Federal Public Service Health, Food chain safety and Environment (RT - Theme 7 – Acron. PORRS) and the European Union (Seventh Framework Programme; Project No. 245141). The funding bodies had no involvement in study design; collection, analysis and interpretation of data; writing of the manuscript; and decision to submit the manuscript for publication.

REFERENCES

Bandrick, M., Pieters, M., Pijoan, C. & Molitor, T. W. (2008). Passive transfer of maternal Mycoplasma hyopneumoniae-specific cellular immunity to piglets. *Clin Vaccine Immunol* **15**, 540-543.

Bautista, E. M., Goyal, S. M., Yoon, I. J., Joo, H. S. & Collins, J. E. (1993). Comparison of porcine alveolar macrophages and CL 2621 for the detection of porcine reproductive and respiratory syndrome (PRRS) virus and anti-PRRS antibody. *J Vet Diagn Invest* **5**, 163-165.

Cano, J. P., Dee, S. A., Murtaugh, M. P. & Pijoan, C. (2007). Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. *Vaccine* **25**, 4382-4391.

Charerntantanakul, W., Platt, R., Johnson, W., Roof, M., Vaughn, E. & Roth, J. A. (2006). Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol* **109**, 99-115.

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., Goyal, S. M., McCullough, S., Morrison, R. B., Joo, H. S. & et al. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest* **4**, 117-126.

Delrue, I., Van Gorp, H., Van Doorsselaere, J., Delputte, P.L. & Nauwynck, H. J. (2010). Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol* **10**, 48.

Geldhof, M. F., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Karniychuk, U. & Nauwynck, H. J. (2012). Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. *BMC Vet Res* **8**, 182.

Halbur, P. G., Paul, P. S., Frey, M. L., Landgraf, J., Eernisse, K., Meng, X. J., Andrews, J. J., Lum, M. A. & Rathje, J. A. (1996). Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet Pathol* **33**, 159-170.

Kapur, V., Elam, M. R., Pawlovich, T. M. & Murtaugh, M. P. (1996). Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. *J Gen Virol* **77**, 1271-1276.

Kim, W. I. & Yoon, K. J. (2008). Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. *Virus Genes* **37**, 380-391.

Kimman, T. G., Cornelissen, L. A., Moormann, R. J., Rebel, J. M. & Stockhofe-Zurwieden, N. (2009). Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. *Vaccine* **27**, 3704-3718.

Labarque, G., Reeth, K. V., Nauwynck, H., Drexler, C., Van Gucht, S. & Pensaert, M. (2004). Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. *Vaccine* **22**, 4183-4190.

Labarque, G. G., Nauwynck, H. J., Van Reeth, K. & Pensaert, M. B. (2000). Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. *J Gen Virol* **81**, 1327-1334.

Li, J., Jiang, P., Li, Y., Wang, X., Cao, J. & Zeshan B. (2009). HSP70 fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus enhanced the immune responses and protective efficacy against virulent PRRSV challenge in pigs. *Vaccine* **27**, 825-832.

Lopez, O. J. & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol* **102**, 155-163.

Lopez, O. J., Oliveira, M. F., Garcia, E. A., Kwon, B. J., Doster, A. & Osorio, F. A. (2007). Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. *Clin Vaccine Immunol* **14**, 269-275.

Martelli, P., Cordioli, P., Alborali, L. G., Gozio, S., De Angelis, E., Ferrari, L., Lombardi, G. & Borghetti, P. (2007). Protection and immune response in pigs intradermally vaccinated against porcine reproductive and respiratory syndrome (PRRS) and subsequently exposed to a heterologous European (Italian cluster) field strain. *Vaccine* **25**, 3400-3408.

Meng, X. J. (2000). Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. *Vet Microbiol* **12**, 309-329.

Meng, X. J., Paul, P. S., Halbur, P. G. & Lum, M. A. (1995). Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. *Arch Virol* **140**, 745-755.

Mengeling, W. L., Vorwald, A. C., Lager, K. M., Clouser, D. F. & Wesley, R. D. (1999). Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **60**, 334-340.

Murtaugh, M. P., Xiao, Z. & Zuckermann, F. (2002). Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol* **15**, 533-547.

Murtaugh, M. P., Elam, M. R. & Kakach, L. T. (1995). Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Arch Virol* **140**, 1451-1460.

Nechvatalova, K., Kudlackova, H., Leva, L., Babickova, K. & Faldyna, M. (2011). Transfer of humoral and cell-mediated immunity via colostrum in pigs. *Vet Immunol Immunopathol* **142**, 95-100.

Nelsen, C. J., Murtaugh, M. P. & Faaberg, K. S. (1999). Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol* **73**, 270-280.

Nelson, E. A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J. E. & Benfield, D. A. (1993). Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. *J Clin Microbiol* **31**, 3184-3189.

Neumann, E. J., Kliebenstein, J. B., Johnson, C. D., Mabry, J. W., Bush, E. J., Seitzinger, A. H., Green, A. L. & Zimmerman, J. J., (2005). Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. *J Am Vet Med Assoc* **227**, 385-392.

Nguyen, T. V., Yuan, L., Azevedo, M. S., Jeong, K. I., Gonzalez, A. M. & Saif, L. J. (2007). Transfer of maternal cytokines to suckling piglets: in vivo and in vitro models with implications for immunomodulation of neonatal immunity. *Vet Immunol Immunopathol* **117**, 236-248.

Nielsen, H. S., Oleksiewicz, M. B., Forsberg, R., Stadejek, T., Bøtner, A. & Storgaard, T. (2001). Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J Gen Virol* **82**, 1263-1272.

Nielsen, J., Bøtner, A., Bille-Hansen, V., Oleksiewicz, M. B. & Storgaard, T. (2002). Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus. *Vet Microbiol* **84**, 1-13.

Nielsen, T. L., Nielsen, J., Have, P., Baekbo, P., Hoff-Jorgensen, R. & Bøtner, A. (1997). Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **54**, 101-112.

Nilubol, D., Platt, K. B., Halbur, P. G., Torremorell, M. & Harris, D. L. (2004). The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. *Vet Microbiol* **102**, 11-18.

Okuda, Y., Kuroda, M., Ono, M., Chikata, S. & Shibata, I. (2008). Efficacy of vaccination with porcine reproductive and respiratory syndrome virus following challenges with field isolates in Japan. *J Vet Med Sci* **70**, 1017-1025.

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F. & Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology* **302**, 9-20.

Papatsiros, V. G., Alexopoulos, C., Kritas, S. K., Koptopoulos, G., Nauwynck, H. J., Pensaert, M. B. & Kyriakis, S. C. (2006). Long-term administration of a commercial porcine reproductive and respiratory syndrome virus (PRRSV)-inactivated vaccine in PRRSV-endemically infected sows. *J Vet Med* **53**, 266-272.

Plana-Duran, J., Bastons, M., Urniza, A., Vayreda, M., Vila, X. & Mane, H. (1997). Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **55**, 361-370.

Salmon, H., Berri, M., Gerdts, V. & Meurens, F. (2008). Humoral and cellular factors of maternal immunity in swine. *Dev Comp Immunol* **33**, 384-393.

Scortti, M., Prieto, C., Alvarez, E., Simarro, I. & Castro, J. M. (2007). Failure of an inactivated vaccine against porcine reproductive and respiratory syndrome to protect gilts against a heterologous challenge with PRRSV. *Vet Rec* **161**, 809-813.

Scortti, M., Prieto, C., Simarro, I. & Castro, J. M. (2006). Reproductive performance of gilts following vaccination and subsequent heterologous challenge with European strains of porcine reproductive and respiratory syndrome virus. *Theriogenology* **66**, 1884-1893.

Thanawongnuwech, R. & Suradhat, S. (2010). Taming PRRSV: Revisiting the control strategies and vaccine design. *Virus Res* **154**, 133-140.

Vanhee, M., Delputte, P. L., Delrue, I., Geldhof, M. F. & Nauwynck, H. J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Vet Res* **40**, 63.

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. & et al. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q* **13**, 121-130.

Zimmerman, J., Benfield, D. A., Murtaugh, M. P., Osorio, F., Stevenson, G. W. & Torremorell, M. (2006). Porcine Reproductive and Respiratory Syndrome Virus (Porcine Arterivirus). In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J., (Eds.), Diseases of Swine 9th Edition. Blackwell Science, USA, pp. 387-417.

Zuckermann, F. A., Garcia, E. A., Luque, I. D., Christopher-Hennings, J., Doster, A., Brito, M. & Osorio, F. (2007). Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol* **123**, 69-85.

CHAPTER 4

VACCINE STRAIN-CULTIVATION AND TESTING ON DIFFERENT CELL LINES

4.1

COMPARISON OF THE EFFICACY OF MARC-145-GROWN INACTIVATED PRRSV VACCINE VIRUS AND PK15$^{Sn\text{-}CD163}$-GROWN INACTIVATED PRRSV VACCINE VIRUS AGAINST HOMOLOGOUS AND HETEROLOGOUS PRRSV CHALLENGE

Marc F. Geldhof, Ivan Trus, Iris Delrue, Jan Van Doorsselaere & Hans J. Nauwynck

Manuscript in preparation

In the field, commercial inactivated and attenuated vaccines are used to control porcine reproductive and respiratory syndrome virus (PRRSV). Nonetheless, clinical symptoms of PRRSV are regularly reported in vaccinating herds. It is generally accepted that there is an urgent need for safe and more effective vaccines that can be adapted regularly to currently circulating isolates. Recently, it was demonstrated that an experimental inactivated MARC-145 grown PRRSV vaccine can be developed that induces virus-neutralizing antibodies upon 2 vaccinations and offers partial protection against homologous challenge. Because all the European and a proportion of the North American PRRSV isolates have to be adapted for growth on MARC-145 cells, which possess only CD163 (not sialoadhesin), unwanted mutations in genes encoding entry-related envelope glycoproteins may occur. Therefore, a PK15$^{Sn\text{-}CD163}$ cell line expressing the two receptors porcine sialoadhesin and porcine CD163 was constructed for PRRSV production. In this study, 2 PRRSV isolates (08V194 and 07V063), were used for PK15$^{Sn\text{-}CD163}$ grown or MARC-145 grown vaccine development and the efficacy of these inactivated PRRSV vaccines was evaluated in homologous and heterologous challenges.

Vaccination of naïve pigs with BEI-inactivated, MARC-145 grown or PK15$^{Sn\text{-}CD163}$ grown PRRSV 08V194 or 07V063 induced a virus-specific antibody response. 08V194-specific neutralizing antibodies were directly induced after double vaccination with both BEI-inactivated, MARC-145 grown viruses (08V194MARCi and 07V063MARCi), while vaccination with BEI-inactivated,

PK15$^{Sn\text{-}CD163}$ grown viruses (08V194PKi and 07V063PKi) only primed; with the latter vaccination approach a faster appearance of virus-neutralizing antibodies upon challenge was observed compared to the non-vaccinated animals. In the groups 08V194MARCi and 08V194PKi, the duration of viremia was significantly reduced with approximately 2 weeks upon homologous challenge (08V194). Similar results were obtained with 07V063PKi upon heterologous challenge (08V194). 07V063MARCi did not significantly influence the viremia upon heterologous challenge (08V194).

Both vaccines, 08V194PKi and 08V194MARCi, provided a significant reduction of viremia upon homologous challenge with 08V194. A similar reduction of viremia was observed in animals vaccinated with 07V063PKi. In contrast, 07V063MARCi had no or only a limited influence on viremia. In addition, groups 08V194MARCi, 08V194PKi and 07V063PKi showed a significant decrease in the number of viremic piglets compared to the mock-vaccinated control group, while no such effect was seen in group 07V063MARCi.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive disorders in sows and boars and is associated with the porcine respiratory disease complex (PRDC), resulting in tremendous economic losses (Christianson et al., 1993; Lager and Mengeling, 1995; Mengeling et al., 1994; Neumann et al., 2005; Terpstra et al., 1991). PRRSV strains are classified into a European genotype (EU type) and a North American genotype (NA type), with the EU prototype Lelystad virus (LV) and the NA prototype VR-2332, which share about 55-70% nucleotide homology (Collins et al., 1992; Nelsen et al., 1999; Wensvoort et al., 1991). In the field, both inactivated and attenuated vaccines are used to control the disease. Attenuated vaccines have the potential to protect animals against viremia, but the protection is dependent on the homology between the vaccine strain and the circulating strain (Labarque et al., 2004). There are also some concerns regarding the safety, as the vaccine strain may still spread and revert to virulence (Dewey et al., 1999; Mengeling et al., 1999; Nielsen et al., 2001; Nielsen et al., 1997). Inactivated vaccines are safe to use, but they are considered less efficacious. They do not provide sufficient protection (Nielsen et al., 1997; Nilubol et al., 2004; Zuckermann et al., 2007). The current incomplete protection of a commercial inactivated vaccine against PRRSV infection may be due to the inactivation procedure used, which can affect the important viral proteins or the antigenic mass (Delrue et al., 2012; Delrue et al., 2009). Viral entry-associated domains are most likely important for virus-neutralizing (VN) antibody induction, since VN antibodies can block infection in macrophages at the stage of entry by inhibiting the interaction of PRRSV with cellular receptors (Delputte et al., 2004). Evidence in favor of a protective role for VN antibodies is found in the correlation between the appearance of VN antibodies in serum and elimination of infectious virus in blood (Labarque et al., 2000). Furthermore, it was shown that passive transfer of VN antibodies gives protection in sows against reproductive failure (Osorio et al., 2002).

PRRS vaccine virus is currently produced in the MARC-145 cell line, as use of these cells overcomes difficulties associated with the use of primary macrophages, the natural host cell of PRRSV. However, since virus entry in MARC-145 cells is different compared to that in primary macrophages, specific domains associated with virus entry could potentially alter during passages in MARC-145 cells (Collins et al.,

1992; Delputte et al., 2004). It is described by others, that after passaging PRRSV strains in MARC-145 cells, mutations in non-structural proteins (nsp1β, nsp2 and nsp10) but also in structural proteins (GP3, GP5 and M) occur (Allende et al., 2000; Indik et al., 2000; Zhou et al., 2009). Mutations in structural viral proteins can cause modifications or loss of neutralizing epitopes resulting in an ineffective humoral immune response towards field strains upon vaccination. To avoid mutations after growth in a cell line and mimicking the entry pathway in macrophages a $PK15^{Sn\text{-}CD163}$ cell line expressing porcine sialoadhesin (Sn) and porcine CD163, two important receptors for entry and infection of macrophages, has been initiated for PRRSV production (Delrue et al., 2010). Sn is a receptor that mediates PRRSV attachment to and internalisation into macrophages (Delputte et al., 2005; Vanderheijden et al., 2003), while CD163 is involved in virus uncoating in macrophages (Van Gorp et al., 2008). Expression of both receptors is shown to be sufficient for PRRSV infection of non-permissive cells (Van Gorp et al., 2008). Once the PK15 cell line, expressing Sn and CD163, was optimized, inactivated vaccines based on recent PRRSV field isolates were developed.

Recently, experimental inactivated MARC-145 grown PRRSV vaccines were developed, based on formerly optimized inactivation procedures (Delrue et al., 2009) and the efficacy of these vaccines was evaluated (Geldhof et al., 2012; Vanhee et al., 2009). In homologous situations, the binary ethyleneimine (BEI) -inactivated PRRSV vaccines have the capacity to induce neutralizing antibodies in naïve animals after 2 vaccinations, resulting in a reduction of viremia after infection (Geldhof et al., 2012; Vanhee et al., 2009). In contrast, the heterologous experimental BEI-inactivated vaccines induce neutralizing antibodies upon vaccination, while no or only a limited effect on the viremic phase upon challenge was observed. The exact reason behind this remains currently unknown and this matter requires further investigation in the future.

Upon booster vaccination with homologous BEI-inactivated vaccines, VN antibodies increased significantly in PRRSV-immune sows. Geldhof et al. (2013) showed that upon vaccination of sows, VN antibodies were transferred to the piglets via colostrum and influenced viremia in piglets, which offers new perspectives for the development of effective and safe PRRSV vaccines.

In this study, 2 recent PRRSV isolates, from outbreaks in herds vaccinated with a registered vaccine, were used for inactivated vaccine development. The main objectives of this study were: (i) to test the capacity of an inactivated PRRSV vaccine, grown on PK15$^{Sn\text{-}CD163}$ cells or grown on MARC-145 cells, to protect naïve pigs upon homologous or heterologous PRRSV challenge and (ii) if the experimental inactivated PRRSV vaccine can be improved if the vaccine virus is produced on PK15$^{Sn\text{-}CD163}$ cells instead of MARC-145 cells.

MATERIALS AND METHODS

Cells and viruses

PRRSV isolates were obtained from 2 farms, showing clinical signs compatible with PRRS in sows or growing pigs despite vaccination against PRRSV. Isolate 07V063 was isolated from fetal tissue, while PRRSV isolate 08V194 was isolated from the serum of a 14-week-old piglet. Both isolates have been used in recent studies by Karniychuk et al. (2011; 2012) and Geldhof et al. (2012), describing viral, clinical and pathological data. Both isolates were isolated and cultured on porcine alveolar macrophages (PAM) and subsequently adapted to MARC-145 cells or PK15$^{Sn\text{-}CD163}$ cells by repeated passages. PAMs were derived from 3-week-old (just weaned) piglets, purchased from a PRRSV- and *Mycoplasma Hyopneumoniae*-negative farm. After isolation, the morphology of PAMs was checked visually via light microscopy. PAMs, MARC-145 cells and PK15$^{Sn\text{-}CD163}$ cells were cultivated as described before (Delrue, 2010). MARC-145 grown stocks of PRRSV 07V063 (2nd passage on PAM + 2 passages on MARC-145) and 08V194 (2nd passage on PAM + 4 passages on MARC-145) and PK15$^{Sn\text{-}CD163}$ cell culture supernatant of PRRSV 07V063 (2nd passage on PAM + 4 passages on PK15$^{Sn\text{-}CD163}$) and 08V194 (2nd passage on PAM + 4 passages on PK15$^{Sn\text{-}CD163}$) were used for vaccine preparation. A 5th passage of 08V194 on PAM, derived from gnotobiotic piglets, was used to challenge the animals.

Virus sequencing of macrophage grown, MARC-145 grown and PK15$^{Sn\text{-}CD163}$ grown 07V063 and 08V194

To determine if adaptation to the MARC-145 cell line resulted in mutations in the structural ORFs, ORF2-7 of MARC-145 grown 07V063 and 08V194 were sequenced and compared with those of original macrophage grown or PK15$^{Sn\text{-}CD163}$ grown 07V063 and 08V194. Sequencing of ORF2-7 of these isolates was performed as described before (Delrue et al., 2010; Geldhof et al., 2012). In summary, RNA was extracted from PRRSV using a RNeasy Protect Mini Kit (QIAGEN) and reverse transcribed using random hexamers and MultiScribe Reverse Transcriptase (Applied Biosystems) according to the manufacturer's guidelines. The primers ORF2a-FW (5'-gtsacaccktatgattacg-3') and ORF2a-REV (5'-tcatrccctattytgcacca-3'), ORF3-FW (5'-agcctacagtacaacaccac-3') and ORF3-REV (5'-agaaaaggcacgcagaaagca-3'), ORF4-FW (5'-cggccaittccatccigag-3') and ORF4-REV (5'- cattcagctcgcataicgtcaag-3'), ORF5-FW2 (5'-tgcticatttcitgacacc-3') and ORF5-REV1 (5'-accttaagigcitatatc-3'), ORF6-FW (5'-taccaactttcttctggac-3') and ORF6-REV (5'-acccagcaactggcacag-3'), ORF7-FW (5'-tggcccctgcccaicacg-3') and ORF7-REV (5'-tcgccctaattgaataggtga-3') were used to amplify the different ORFs with Taq Polymerase (Invitrogen, Merelbeke, Belgium). PCR products were treated with Exonuclease I and Antarctic Phosphatase (New England Biolads, Ipswich, USA) and used directly for cycle sequencing with a Big Dye Terminator Cycle sequencing kit V1.1 (Applied Biosystem, Foster City, USA) and PRRSV primers. Cycle sequencing reaction products were purified by ethanol precipitation and separated on an ABI Genetic 310 (Applied Biosystem, Foster City, USA). The amino acid (aa) sequences were analyzed and compiled by BlastN and BlastP (www.ncbi.nlm.nih.gov), and Sixframe, ClustalW, Align (workbench.sdsc.edu). Nucleotide sequences were submitted to Genbank under accession numbers [GenBank: GU737264] (07V063) and [GenBank: GU737265] (08V194).

Virus purification, inactivation and quality control

MARC-145 grown and PK15$^{Sn\text{-}CD163}$ grown supernatant containing the virus were purified via ultracentrifugation as previously described by Vanhee et al. (2009). Purified virus (07V063 and 08V194) was suspended in RPMI 1640 (Invitrogen) to a titer of 10^9 $TCID_{50}$/mL on macrophages (equal to a titer of 10^8 $TCID_{50}$/mL on

MARC-145 cells). Subsequently, the virus was inactivated using BEI as described before (Vanhee et al., 2009) and inactivated virus was stored at -70 °C. To confirm that all viruses were completely inactivated, a complete vaccine dose of 08V194 (MARC-145 grown and PK15$^{Sn\text{-}CD163}$ grown) and 07V063 (MARC-145 grown and PK15$^{Sn\text{-}CD163}$ grown) was inoculated on MARC-145 cells and subsequently passaged twice. As a positive control, MARC-145 cells were inoculated with 1 mL of non-inactivated 07V063 and 08V194. The MARC-145 cells were routinely checked for cytopathic effect (CPE) and ultimately stained for the PRRSV nucleocapsid protein via an immunoperoxidase staining using monoclonal antibody 13E2 (Van Breedam et al., 2011). No CPE or positive nucleocapsid staining was detected in cells that were inoculated with inactivated virus, while clear CPE and nucleocapsid staining were observed in cell cultures that were inoculated with non-inactivated virus.

Since conservation of entry of inactivated virus may serve as a quality control for the preservation of antigenic properties, the effect of BEI-inactivation on virus attachment and internalization into macrophages was examined as described previously (Delrue et al., 2010; Vanhee et al., 2009). Non-inactivated virus suspensions were included as positive controls. The entry experiment showed that the binding and internalization kinetics of all BEI-inactivated virus stocks are similar to those observed for the non-inactivated virus stocks. Western blotting tested the viral antigen load for all vaccines.

Experimental design

Thirty-one four-week-old piglets were purchased from a PRRSV-negative farm and their PRRSV-seronegative status was confirmed by IPMA upon arrival. The animals were housed in isolation units with HEPA-filtered air and kept during 7 days to allow adaptation to the new conditions and were randomly divided into 6 groups (Table 1). An oil-in-water (o/w) diluent, normally used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky (Fort Dodge Animal Health), was used as an adjuvant and is further referred to as o/w Suvaxyn. A first group (group CON, n = 7 pigs) served as a mock-vaccinated control group and received 1 mL RPMI 1640 in 1 mL o/w Suvaxyn intramuscularly at 5 and 9 weeks of age. Four other groups were vaccinated twice intramuscularly at 5 (primo vaccination) and 9 (booster vaccination) weeks of age. Group 08V194MARCi (n = 6 pigs) was vaccinated with 1 mL BEI-inactivated MARC-145 grown 08V194 (10^9 $TCID_{50}$ on macrophages) in 1 mL o/w Suvaxyn and

group 08V194PKi (n = 6) was vaccinated with BEI-inactivated PK15$^{Sn\text{-}CD163}$ grown 08V194 (10^9 $TCID_{50}$ on macrophages) in 1 mL o/w Suvaxyn. Group 07V063MARCi (n = 6 pigs) was vaccinated with 1 mL BEI-inactivated MARC-145-grown 07V063 (10^9 $TCID_{50}$ on macrophages) in 1 mL o/w Suvaxyn and group 07V063PKi (n = 6 pigs) received 1 mL BEI-inactivated PK15$^{Sn\text{-}CD163}$ grown 07V063 (10^9 $TCID_{50}$ on macrophages) in 1 mL o/w Suvaxyn. BEI-inactivated vaccines were administered intramuscularly in the neck muscles behind the ear. At 13 weeks of age, all pigs were challenged intranasally with PRRSV 08V194 (10^6 $TCID_{50}$) in phosphate buffered saline (PBS) (2,5 ml per nostril). General health, appetite and rectal body temperature of the pigs were monitored daily. Blood samples were taken by jugular venipuncture weekly after (primo) vaccination and at 0, 1, 3, 5, 7, 10, 14, 21, 28, 35 and 42 days post challenge (dpc). Serum was collected and stored at -70 °C. Serum samples for IPMA and VN antibody detection were incubated for 30 min at 56 °C prior to freezing.

The local ethical committee of the Faculty of Veterinary Medicine, Ghent University, approved the animal experiments.

Table 1: Experimental design of vaccination-challenge experiment

Group	Vaccination	Cell line used for vaccine production	Age in weeks	Challenge strain (13 weeks)
CON	Mock	-	5 and 9	08V194
08V194MARCi	BEI-inactivated 08V194	MARC-145 cells	5 and 9	08V194
08V194PKi	BEI-inactivated 08V194	PK15$^{Sn\text{-}CD163}$ cells	5 and 9	08V194
07V063MARCi	BEI-inactivated 07V063	MARC-145 cells	5 and 9	08V194
07V063PKi	BEI-inactivated 07V063	PK15$^{Sn\text{-}CD163}$ cells	5 and 9	08V194

Serological examinations and virus titration

Serum samples were examined for the presence of PRRSV-specific antibodies using an IPMA as described by Labarque et al. (2000). IPMA tests were performed on 08V194-infected MARC-145 cells. VN antibodies were detected by a seroneutralization (SN) assay on MARC-145 cells as described before (Geldhof et al.,

2012). SN tests were performed using the virus variant 08V194. Each serum sample was tested in duplicate. Briefly, serum samples were twofold serially diluted and an equal volume of a PRRSV 08V194 (2^{nd} passage on PAM + 4 passages on MARC-145) suspension (titer 2 x 10^3 $TCID_{50}$/ mL) was added to each dilution. After mixing, the plates were incubated at 37 °C for 1 h and 50 µl of the mixture was subsequently transferred to confluent monolayers of MARC-145 cells in 96-well plates. Cells were screened for 7 days after inoculation and the neutralization titer of the sera was recorded as the reciprocal of the highest dilution that inhibited CPE in 50% of the inoculated wells. To check the sensitivity to PRRSV infection of different passages of MARC-145 cells, control titrations using PRRSV stocks (isolate 07V063 and isolate 08V194) with a known virus titer were performed in parallel with each neutralization assay. Virus titers in serum were determined by virus titration on 24 h-cultivated PAM following a standard procedure (Labarque et al., 2000). 72 hours post inoculation, cells were fixed and an immunoperoxidase staining with monoclonal antibody 13E2 against the PRRSV nucleocapsid protein was performed to visualize infection in the cells (Van Breedam et al., 2011). The titers were calculated as described by Reed and Muench (1938) and expressed as $TCID_{50}$/mL. To check the sensitivity of the PAM, all cell batches were assayed in virus titrations using a PRRSV stock (08V194) with a known virus titer.

Statistical analyses

Antibody titers and virus titers were analyzed by Kruskall-Wallis test, followed by Dunn's multiple comparisons test to determine significant differences with the control group at different time points. Samples, that tested negative in IPMA, VN or virus isolation, were consequently given a numerical value of 0.0. A two-tailed Fisher's exact test was used to determine significant differences between the number of viremic animals in the vaccinated groups and the control group at different time points. An overall *p* value of 0.05 was taken as the level of statistical significance. All statistical analyses were performed using GraphPad Prism version 5.0a (GraphPad Software, San Diego, California, USA).

RESULTS

Virus sequencing

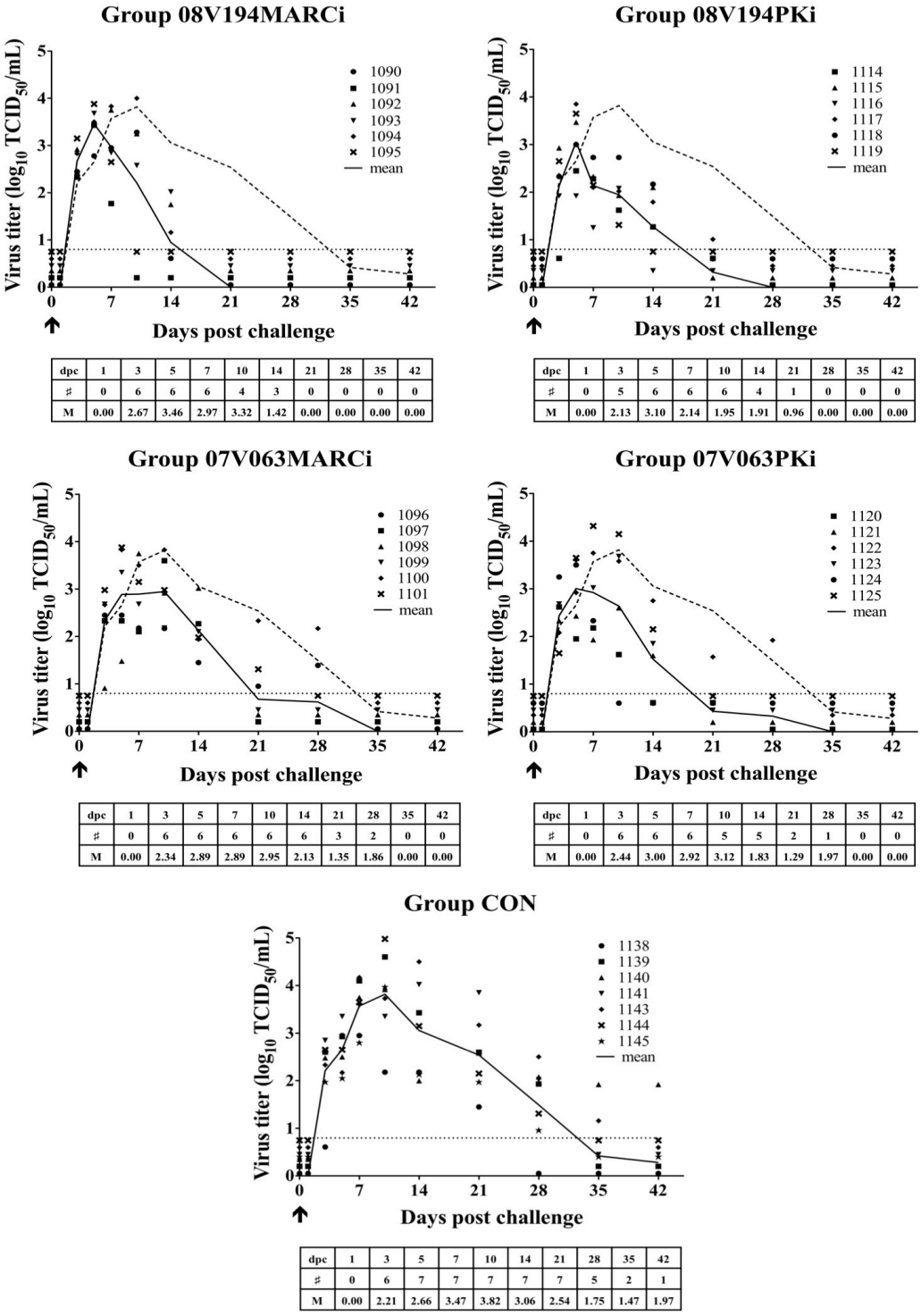
The aa sequences of structural proteins ORF2-7 of MARC-145 grown and PK15$^{Sn-CD163}$ grown 07V063 or 08V194 were 100% identical to those of the corresponding proteins of original macrophage grown virus. The clear difference in aa sequence between both 07V063 and 08V194 and with the EU prototype LV and the NA prototype VR-2332 allowed their classification as EU wild-type viruses that are not of vaccine origin. The ORF5 homology of 08V194 and 07V063 with LV is 86% and 90%, respectively. The ORF5 sequences of the 2 strains 08V194 and 07V063 show 86% homology.

Clinical examination

None of the groups showed any relevant clinical sign at any time during the vaccination period or after challenge exposure. The daily rectal temperatures varied in all groups and no statistically significant differences were observed. Challenge with PRRSV isolate 08V194 induced moderate fever (higher than 39.5 °C, but not higher than 40.5°C) within 7 days post infection in 23 out of 31 inoculated pigs. The 8 remaining animals did not develop fever. By 9 days post challenge, fever had disappeared in all animals.

Viremia

Upon challenge, all animals became viremic. In group CON, a maximum mean virus titer of 3.8 $\log_{10}$ $TCID_{50}$/mL was reached at 10 dpc. Subsequently, a decline in virus titer was observed and virus was no longer detectable in the serum at 4, 5 or 6 weeks after challenge, depending on the animal. Still, 1 piglet remained virus positive till 6 weeks post challenge (Fig. 1, CON). In group 08V194MARCi, the viremic peak at day 5 was not reduced, but the mean virus titer at day 14 was significantly reduced and from 21 dpc, virus could no longer be detected in any of the piglets (Fig. 1, 08V194MARCi). The number of viremic piglets in group 08V194MARCi was significantly lower compared to group CON on day 21 and 28 post challenge. The mean virus titer in group 08V194PKi reached 3.1 $\log_{10}$ $TCID_{50}$/mL at 5 dpc, but virus titers were not significantly different from those in group CON. At time points 21 and



dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	6	6	4	3	0	0	0	0
M	0.00	2.67	3.46	2.97	3.32	1.42	0.00	0.00	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	0	5	6	6	6	4	1	0	0	0
M	0.00	2.13	3.10	2.14	1.95	1.91	0.96	0.00	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	6	6	6	6	3	2	0	0
M	0.00	2.34	2.89	2.89	2.95	2.13	1.35	1.86	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	6	6	5	5	2	1	0	0
M	0.00	2.44	3.00	2.92	3.12	1.83	1.29	1.97	0.00	0.00

dpc	1	3	5	7	10	14	21	28	35	42
#	0	6	7	7	7	7	7	5	2	1
M	0.00	2.21	2.66	3.47	3.82	3.06	2.54	1.75	1.47	1.97

Figure 1. Serum-virus titers after challenge for group CON (Mock-vaccinated control), 08V194MARCi (BEI-inactivated, MARC-145 grown 08V194), 08V194PKi (BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 08V194), 07V063MARCi (BEI-inactivated, MARC-145 grown 07V063) and 07V063PKi (BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 07V063). Virus titers in serum (log_{10} $TCID_{50}$/mL) were determined by virus titration on PAM, followed by immunoperoxidase staining for the PRRSV nucleocapsid protein. ↑ = challenge. Symbols represent individual animals and solid lines represent mean virus titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON. The dotted line marks the detection limit for virus titration. Mentioned in the table: # = the number of viremic animals in the different groups at different time points. M = mean virus titer of all viremic animals in the group at different time points.

28 dpc however, virus titers were significantly reduced compared to group CON. Moreover, viremia in group 08V194PKi was already cleared at 21 dpc for 5 animals and at 28 dpc, all animals were negative (Fig. 1, 08V194PKi). From 21 till 28 dpc, the total number of viremic animals in group 08V194PKi was significantly lower than in group CON. Mean virus titers in group 07V063MARCi were comparable to those in group 07V063PKi, reaching a peak around 5-10 dpc with a maximum mean titer of 2.8 $\log_{10}$ $TCID_{50}$/mL and 2.9 $\log_{10}$ $TCID_{50}$/mL, respectively. No significant differences could be detected at any time point between group 07V063MARCi and group CON. For 3 animals of this group, virus was cleared from blood at 3 weeks, for 1 other at 4 weeks and in the remaining animals at 5 weeks post challenge (Fig. 1, 07V063MARCi). In group 07V063PKi, viremia was detected in all animals, with a peak around 5-7 dpc. The viremic phase showed a similar pattern as for group 07V063MARCi, but in this group viremia was significantly reduced on day 21 and 28 post challenge compared to group CON. Viremia disappeared in all animals by 5 weeks after challenge (Fig. 1, 07V063PKi). The number of viremic piglets in group 07V063PKi was significantly lower compared to group CON on day 21 and 28 post challenge. In summary, groups 08V194MARCi, 08V194PKi and 07V063PKi showed a significant reduced viremia in time and a significant decrease in the number of viremic piglets compared to the mock-vaccinated group CON, while no such effect was seen in group 07V063MARCi.

08V194 virus-specific antibodies

All mock-vaccinated animals (group CON) had virus-specific serum antibodies starting from 7 dpc (Fig. 2, CON). All 6 animals that were vaccinated with BEI-inactivated, MARC-145 grown 08V194 virus (group 08V194MARCi) seroconverted at 2 or 3 weeks after the first vaccination. Similarly, 5 out of 6 animals that were vaccinated with BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown 08V194 (group 08V194PKi) showed virus-specific antibodies at 2 or 3 weeks after the first vaccination. In the BEI-inactivated, MARC-145 grown 07V063 vaccinated group (group 07V063MARCi), 08V194-specific antibodies were detected from 2 weeks after primo vaccination and all animals seroconverted after booster vaccination. In the group vaccinated with BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown 07V063 (group 07V063PKi) 5 out 6 pigs showed positive antibody titers at 2 or 3 weeks after the first vaccination

and all animals seroconverted after booster vaccination. Antibody titers in all 4 vaccinated groups were significantly higher compared to group CON from 1 week after booster vaccination up till 21 dpc (except for time point 10 and 21 dpc in group 08V194PKi and group 07V063PKi, respectively) (Fig. 2, 08V194MARCi, 08V194PKi, 07V063MARCi and 07V063PKi). After 21 dpc, mean antibody titers in groups 08V194MARCi, 08V194PKi, 07V063MARCi and 07V063PKi remained higher compared to the control group, although differences were not significant. In summary, the course of the IPMA antibody titers in all groups was similar to those described in previous studies and the former experiments in this thesis (Diaz et al., 2005; Meier et al., 2003; Yoon et al., 1995).

08V194-specific virus-neutralizing antibodies

Starting from 21 dpc, 3 pigs of group CON showed a VN antibody titer and by 35 dpc, VN antibodies appeared in all mock-vaccinated pigs (Fig. 3, CON). All 6 pigs of group 08V194MARCi showed high VN antibody titers at 1 week after the booster vaccination and this remained so until the end of the experiment (Fig. 3, 08V194MARCi). VN antibody titers were significantly higher in group 08V194MARCi compared to group CON from 1 week after booster vaccination until 5 weeks post challenge, with mean values ranging from 3.2-6.2 $\log_2$. Three animals of group 08V194PKi seroconverted for VN antibodies at least once within 3 weeks after booster vaccination, but mean VN antibody titers remained low and were only significantly higher than group CON at 7 till 21 dpc, reaching mean values ranging from 4.3-7.7 $\log_2$ (Fig. 3, 08V194PKi). A similar pattern as in group 08V194MARCi was observed in group 07V063MARCi, where 08V194-neutralizing antibodies could already be detected at 1 week after booster vaccination. Two animals turned negative for VN antibodies in the period between 2 weeks post booster vaccination and 10 dpc, but after this period, VN antibodies were consistently detected in all 6 animals. The mean VN antibody titer in group 07V063MARCi was significantly higher compared to group CON in the period between 1 week after booster vaccination and 4 weeks post challenge (except for time point 5 dpc), reaching mean values ranging from 2.1-4.7 $\log_2$ (Fig. 3, 07V063MARCi). None of the animals in group 07V063PKi showed 08V194-specific VN antibodies before challenge, but VN antibodies already appeared between 5 and 10 dpc. The mean VN antibody titer in group 07V063PKi was slightly

but not significantly higher compared to group CON between 7 and 21 dpc, reaching a maximum of 3.5 log_2 at 14 dpc (Fig. 3, 07V063PKi). In summary, both 08V194MARCi and 07V063MARCi vaccine viruses induced a stronger 08V194-specific VN antibody response upon booster vaccination, than both 08V194PKi and 07V063PKi vaccine viruses. A faster appearance of VN antibodies post challenge was observed in both groups vaccinated with the $PK15^{Sn\text{-}CD163}$-grown virus.

DISCUSSION

In this study, 2 circulating PRRSV strains were used for vaccine development. We investigated the capacity of an inactivated PRRSV vaccine, grown on $PK15^{Sn\text{-}CD163}$ cells or grown on MARC-145 cells, to protect naïve pigs upon homologous or heterologous PRRSV challenge. We further examined if the experimental inactivated PRRSV vaccine can be improved if the vaccine virus is produced on $PK15^{Sn\text{-}CD163}$ cells instead of MARC-145 cells. Since the $PK15^{Sn\text{-}CD163}$ cells express Sn and CD163, it is expected that no or less mutations in the structural viral proteins will occur, since natural entry is mimicked. For both PRRSV isolates used in the study, it is shown by sequencing that mutation of structural viral proteins did not occur after growth on $PK15^{Sn\text{-}CD163}$ cells. It is important that no mutations occur in the ORFs encoding the structural viral proteins, since these are important for the induction of a VN antibody response and protection against infection. Mutations in ORFs encoding structural viral proteins after growth on $PK15^{Sn\text{-}CD163}$ cells cannot always be avoided for all PRRSV strains (Delrue et al., 2010).

The pigs of all 4 vaccinated groups showed a virus-specific antibody response upon booster vaccination, while the pigs in the control group had no detectable virus-specific antibody titers before challenge. After challenge, all pigs in the vaccinated groups reached higher or similar virus-specific antibody titers than the pigs in the control group. These results are in line with those described in previous studies and the former experiments in this thesis (Diaz et al., 2005; Meier et al., 2003; Yoon et al., 1995).

Vaccination with 07V063MARCi or 08V194MARCi vaccines consistently induced sizable titers of PRRSV-neutralizing antibodies after at least 2 immunizations given four weeks apart.

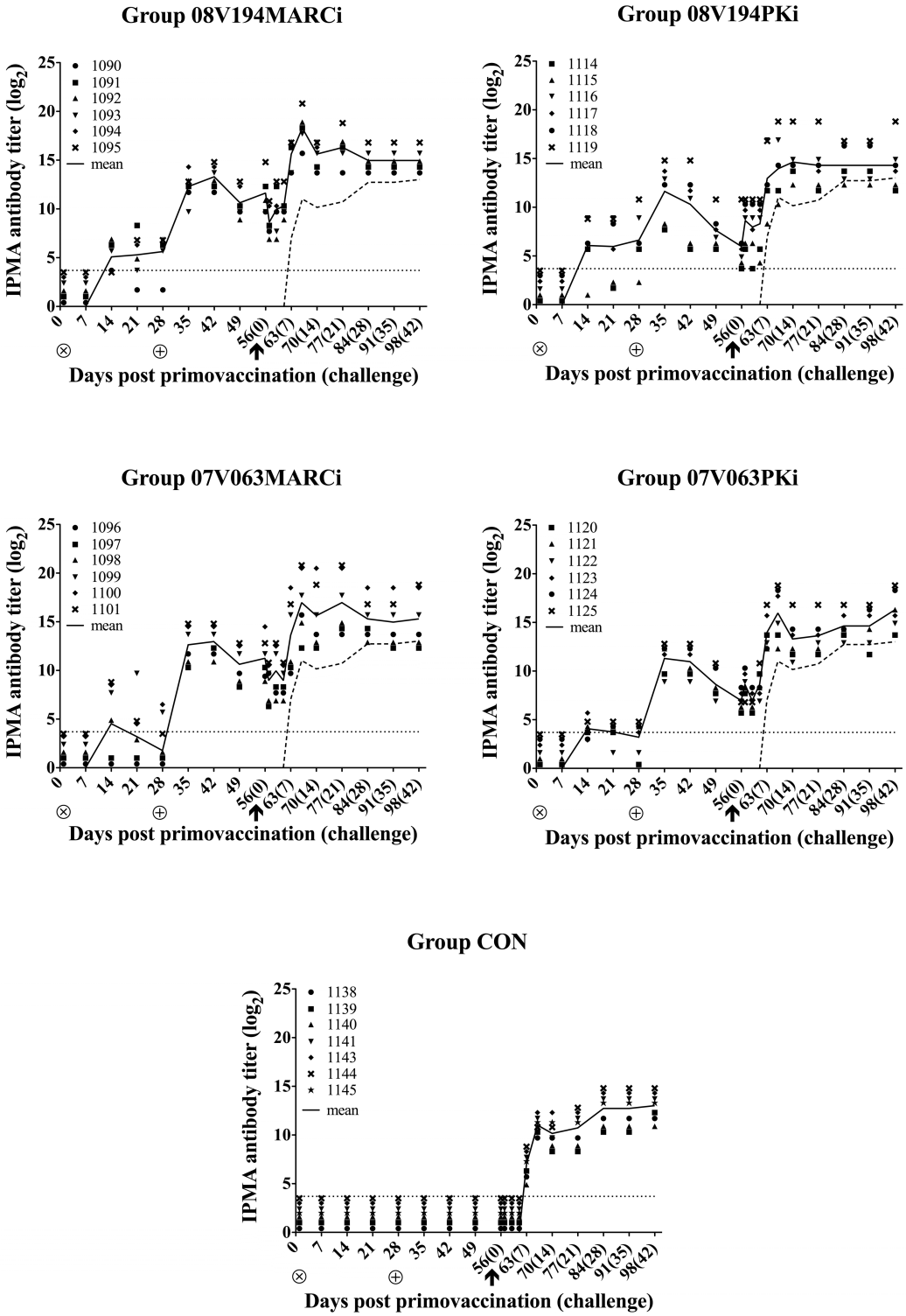


Figure 2. PRRSV-specific IPMA antibody titers (log_2) after vaccination and challenge for group CON (Mock-vaccinated control), 08V194MARCi (BEI-inactivated, MARC-145 grown 08V194), 08V194PKi (BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 08V194), 07V063MARCi (BEI-inactivated, MARC-145 grown 07V063) and 07V063PKi (BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 07V063). ⊗ = primo vaccination; ⊕ = booster vaccination; ↑ = challenge. Symbols represent individual animals and solid lines represent mean IPMA titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON. The dotted line marks the detection limit for the IPMA test.

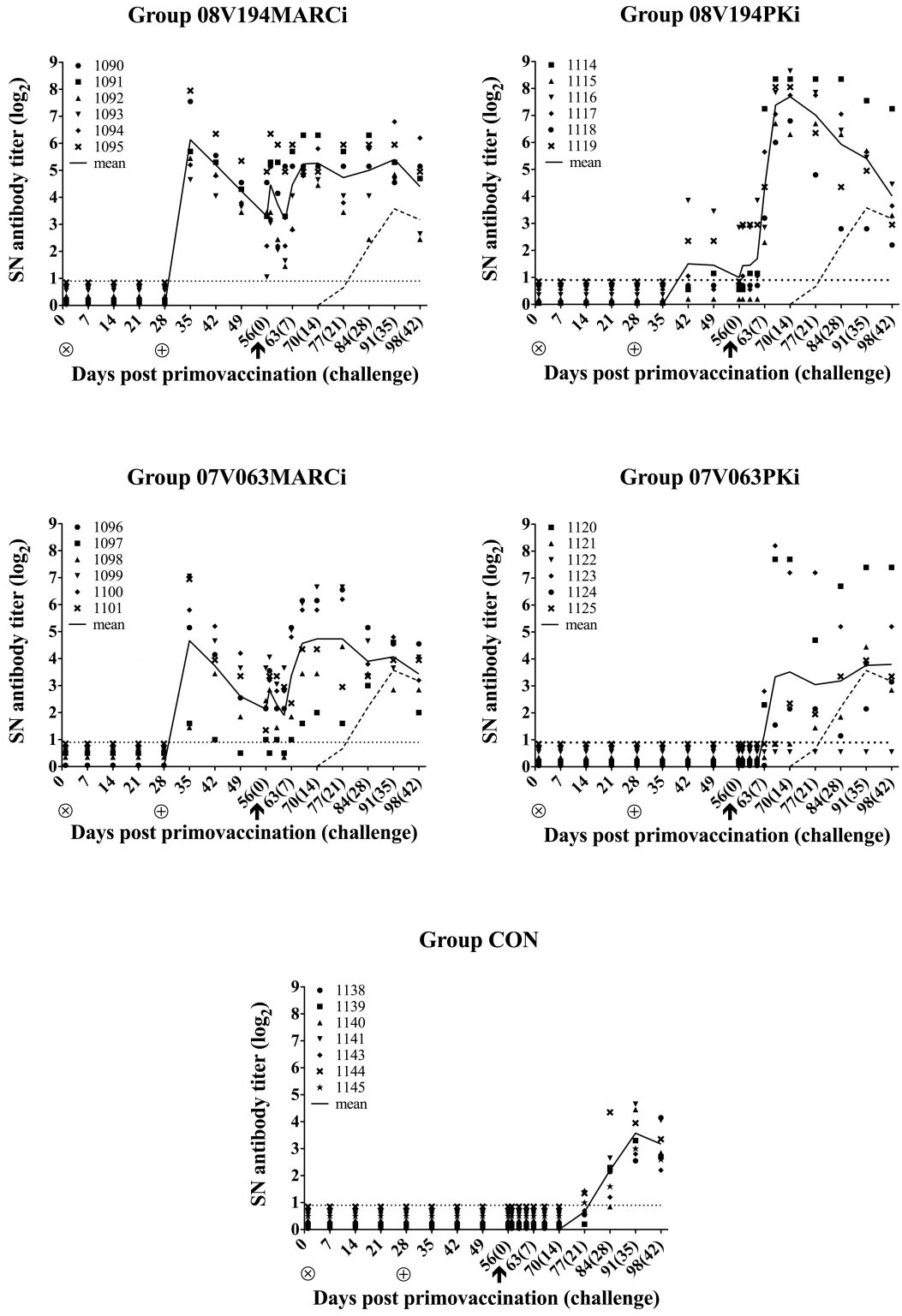


Figure 3. PRRSV-neutralizing antibody titers (log_2) after vaccination and challenge for group CON (Mock-vaccinated control), 08V194MARCi (BEI-inactivated, MARC-145 grown 08V194), 08V194PKi (BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 08V194), 07V063MARCi (BEI-inactivated, MARC-145 grown 07V063) and 07V063PKi (BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 07V063). ⊗ = primo vaccination; ⊕ = booster vaccination; ↑ = challenge. Symbols represent individual animals and solid lines represent mean SN titers calculated on all animals present in each group. The dashed line indicates the mean titers for all animals in group CON. The dotted line marks the detection limit for the SN test.

The 08V194PKi vaccine also induced 08V194-neutralizing antibodies upon double vaccination, with the induced titers being lower than the 08V194-induced titers in both 07V063MARCi and 08V194MARCi groups. In all 3 groups, the VN titers dropped immediately after challenge, which may indicate that they were consumed during their interaction with virus early in infection. However, after this initial drop in VN antibody titers, VN antibodies quickly reappeared in higher amounts.

In the pigs in the group 07V063PKi the VN antibody response was only detectable upon challenge, but was strongly primed compared to the animals in group CON. The fast appearance of VN antibodies upon challenge for MARC-145 grown and for PK15$^{Sn\text{-}CD163}$ grown vaccines is in agreement with the findings in previous studies (Delrue, 2010; Misinzo et al., 2006; Vanhee et al., 2009). Moreover, it demonstrates the potential of priming the neutralizing antibody response by immunization with a high dose of inactivated PRRSV. In addition, as described by other authors, (Labarque et al., 2003; Molitor et al., 1997; Vanhee et al., 2009) a correlation between the appearance of high VN antibody titers and the reduction of viremia was observed, indicating that VN antibodies may contribute to protection against the virus. At this point, no evidence was available showing that PK15$^{Sn\text{-}CD163}$ grown vaccines were able to induce VN antibodies upon double vaccination, as determined by the SN test on MARC-145 cells. In a previous study (Delrue et al., 2010, unpublished data), VN antibodies before challenge in the groups vaccinated with the PK15$^{Sn\text{-}CD163}$ grown virus were absent. This discrepancy can be due to the sensitivity of the classical SN test, in which MARC-145 cells and MARC-145 grown virus is used. It is possible that antibodies against virus grown on PK15$^{Sn\text{-}CD163}$ cells cannot or less efficiently neutralize MARC-145 grown virus. Therefore an SN test on PK15$^{Sn\text{-}CD163}$ cells or macrophages with PK15$^{Sn\text{-}CD163}$ grown or macrophage grown virus would be more relevant. The SN test on PK15$^{Sn\text{-}CD163}$ cells should be optimized and all serum samples of this study should be tested. It is also possible that antibodies with a low avidity are missed in the current SN test, since the serum-virus mixture is kept on the cells for 10 days. When serum and virus are mixed, VN antibodies will bind to the virus, but after 10 days, it may be that VN antibodies with a low avidity binding to the virus, detach from the virus after a short time. Washing the serum-virus mixture away from the cells after 1 hour can probably solve this problem. Third, the pool of VN antibodies induced by MARC-145 grown virus might be different from the pool

induced by PK15$^{Sn\text{-}CD163}$ grown virus due to the divergent entry pathway in MARC-145 cells and PK15$^{Sn\text{-}CD163}$ cells.

Despite the significant role that VN antibodies seem to play in protection, the induction of homologous VN antibodies was not sufficient to completely protect the animals, as it still permitted the development of a viremia post-challenge that lasted at least 2 weeks. A possible explanation is that a high dose of virus (10^6 $TCID_{50}$) was used for challenge and that higher VN antibody titers may be needed at the time of challenge to offer full protection. Based on data by Benfield et al. (2000) the $TCID_{50}$ for field exposure via intranasal route is approximately 10^3-10^4 $TCID_{50}$. Although it has been reported that the PRRSV-specific neutralizing antibody response is to a large extent strain specific and a lack in cross-neutralization may occur even between genetically closely related virus strains (Kim & Yoon, 2008; Okuda et al., 2008), our data indicates that between the 2 EU type viruses used in this study, cross-neutralization exists. The 07V063MARCi and 07V063PKi vaccines used in this study induced antibodies that could neutralize the heterologous challenge virus in *in vitro* SN assays and consequently could limit viremia under heterologous challenge conditions. These results suggest that in PRRSV, under some circumstances heterologous VN antibodies can be equally efficient as the homologous VN antibodies. This was recently observed by Martinez-Lobo et al. (2011). Differences in cross-reactivity might be due to different reasons. A first factor is the antigenic variability of neutralizing epitopes (NE). Although different viral proteins, including GP2, GP3, GP4, GP5 and M protein, have been identified as inducers of VN antibodies by different approaches, the NEs of PRRSV have not been fully characterized (Ansari et al., 2006; Cancel-Tirado et al., 2004; Kim & Yoon, 2008; Kim et al., 2007; Vanhee et al., 2011). Changes in the aa sequence of these NEs might prevent recognition of those epitopes by VN antibodies. A second factor that can change the VN profile is glycosylation of envelope proteins that is used by enveloped viruses as a mechanism of immune evasion used to escape, block or minimize virus VN antibody response making epitopes poorly immunogenic (Ansari et al., 2006). Still, the nature of this phenomenon remains currently unknown. Clearly, this matter requires further investigation in the future.

Both 08V194MARCi and 08V194PKi vaccines were effective in partially protecting

naïve pigs upon homologous challenge. They reduced the duration of viremia with approximately 3 weeks upon homologous challenge, when compared to the viremic phase in the respective mock-vaccinated group, which lasted approximately 1 month. Vaccines 07V063MARCi and 07V063PKi were included to study the strain variability in relation to its impact on vaccine efficacy. We found a significant reduction of viremia upon vaccination with 07V063PKi and a non-significant reduction in 08V194 viremia after the use of 07V063MARCi. These results are in line with earlier studies published by Vanhee et al. (2009) and Geldhof et al. (2012), showing that BEI-inactivated vaccines can be successful in reducing viremia upon homologous challenge. This is also the first report of an inactivated, $PK15^{Sn\text{-}CD163}$ grown PRRSV vaccine that manages to induce partial protection upon homologous and heterologous challenge. Nevertheless, we have to take in account that when we look to the graphs and results no biologically relevant differences are visible. Considering the similar efficacy in homologous situations of MARC-145 grown or $PK15^{Sn\text{-}CD163}$ grown vaccines virus, it can be questioned whether the use of $PK15^{Sn\text{-}CD163}$ grown vaccines is advantageous over the use of MARC-145 grown vaccines. Hurdles concerning the use of MARC-145 cells are the different entry pathway for PRRSV to grow on MARC-145 cells (Collins et al., 1992; Tan et al., 2001) and patents that restrict the commercial use of these cells. Another issue, most field isolates do not replicate in MARC-145 cells, with a report showing that only 2% of the tested field samples replicated in MARC-145 cells, contrasting to 70% in PAM (de Abin et al., 2009). Recent preliminary results in our laboratory showed that primary PRRSV field isolates are able to grow immediately without passage on $PK15^{Sn\text{-}CD163}$ cells.

Above-mentioned results suggest that both MARC-145 grown and $PK15^{Sn\text{-}CD163}$ grown, inactivated vaccines may provide a more or less standardized, predictable degree of protection upon homologous challenge. Still, the production process of inactivated vaccines may appear too elaborate and costly and further optimization to improve MARC-145 grown or $PK15^{Sn\text{-}CD163}$ grown, inactivated PRRSV vaccines remains a challenge for the future. An important question is the dose that is needed for inducing a protective immunity. If it is possible to skip the purification/concentration step, this type of vaccine is commercially interesting, even for the production of autogenous vaccines. This aspect will be studied in the near

future. Further research is needed to explore the value of this promising PK15$^{Sn\text{-}CD163}$ cell line, thereby focusing on virus isolation and production.

ACKNOWLEGDEMENTS

The authors would like to thank Chantal Vanmaercke, Carine Boone, Melanie Bauwens, Nele Dennequin, Dries Helderweirt, Geert Opsomer, Bart Ellebaut and Zeger Vandenabeele for their highly appreciated and excellent technical assistance. This work was funded by the Federal Public Service Health, Food chain safety and Environment (RT - Theme 7 – Acron. PORRS) and the European Union (Seventh Framework Programme; Project No. 245141).

REFERENCES

Allende, R., Laegreid, W. W., Kutish, G. F., Galeota, J. A., Wills, R. W. & Osorio, F. A. (2000). Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. *J Virol* **74**, 10834-10837.

Ansari, I. H., Kwon, B., Osorio, F. A. & Pattnaik, A. K. (2006). Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol* **80**, 3994-4004.

Benfield, D. A., Nelson, C., Steffen, M. & Rowland, R. R. R. (2000). Transmission of PRRSV by artificial insemination using extended semen seeded with different concentrations of PRRSV. *Proc Annu Meet Am Assoc Swine Pract* 405-408.

Cancel-Tirado, S. M., Evans, R. B. & Yoon, K. J. (2004). Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. *Vet Immunol Immunopathol* **102**, 249-262.

Christianson, W. T., Choi, C. S., Collins, J. E., Molitor, T. W., Morrison, R. B. & Joo, H. S. (1993). Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. *Can J Vet Res* **57**, 262-268.

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., Goyal, S. M., McCullough, S., Morrison, R. B., Joo, H. S. & et al. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest* **4**, 117-126.

de Abin, M. F., Spronk, G., Wagner, M., Fitzsimmons, M., Abrahante, J. E. & Murtaugh, M. P. (2009). Comparative infection efficiency of porcine reproductive and respiratory syndrome virus field isolates on MA104 cells and porcine alveolar macrophages. *Can J Vet Res* **73**, 200-204.

Delputte, P. L., Costers, S. & Nauwynck, H. J. (2005). Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. *J Gen Virol* **86**, 1441-1445.

Delputte, P. L., Meerts, P., Costers, S. & Nauwynck, H. J. (2004). Effect of virus-specific antibodies on attachment, internalization and infection of porcine reproductive and respiratory syndrome virus in primary macrophages. *Vet Immunol Immunopathol* **102**, 179-188.

Delrue, I., Verzele, D., Madder, A. & Nauwynck, H. J. (2012). Inactivated virus vaccines from chemistry to prophylaxis: merits, risks and challenges. *Expert Rev Vaccines* **11**, 695-719.

Delrue, I., Van Gorp, H., Van Doorsselaere, J., Delputte, P. L. & Nauwynck, H. J. (2010). Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol* **10**, 48.

Delrue, I. (2010). Development of a new inactivated porcine reproductive and respiratory syndrome virus (PRRSV) vaccine. *Phd Thesis* 122-141.

Delrue, I., Delputte, P. L. & Nauwynck, H. J. (2009). Assessing the functionality of viral entry-associated domains of porcine reproductive and respiratory syndrome virus during inactivation procedures, a potential tool to optimize inactivated vaccines. *Vet Res* **40**, 62.

Dewey, C. E., Wilson, S., Buck, P. & Leyenaar, J. K., (1999). The reproductive performance of sows after PRRS vaccination depends on stage of gestation. *Prev Vet Med* **40**, 233-241.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. (2005). Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. *J Gen Virol* **86**, 1943-1951.

Geldhof, M. F., Van Breedam, W., De Jong, E., Lopez Rodriguez, A., Karniychuk, U. U., Vanhee, M., Van Doorsselaere, J., Maes, D. & Nauwynck, H. J. (2013). Antibody response and maternal immunity upon boosting PRRSV-immune sows with experimental farm-specific and commercial PRRSV vaccines. *Vet Mic* conditionally accepted

Geldhof, M. F., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Karniychuk, U. & Nauwynck, H. J., (2012). Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. *BMC Vet Res* **8**, 182.

Indik, S., Valicek, L., Klein, D. & Klanova, J. (2000). Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **81**, 2497-2502.

Karniychuk, U. U., Saha, D., Vanhee, M., Geldhof, M. F., Cornillie, P., Caij, A. B., De Regge, N. & Nauwynck, H. J. (2012). Impact of a novel inactivated PRRSV vaccine on virus replication and virus-induced pathology in fetal implantation sites and foetuses upon challenge. *Theriogenology* **78**, 1527-1537.

Karniychuk, U. U., Saha, D., Geldhof, M., Vanhee, M., Cornillie, P., Van den Broeck, W. & Nauwynck, H. J. (2011). Porcine reproductive and respiratory syndrome virus (PRRSV) causes apoptosis during its replication in fetal implantation sites. *Microb Pathog* **51**, 194-202.

Kim, W. I. & Yoon, K. J. (2008). Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. *Virus Genes* **37**, 380-391.

Kim, W. I., Lee, D. S., Johnson, W., Roof, M., Cha, S. H. & Yoon, K. J. (2007). Effect of genotypic and biotypic differences among PRRS viruses on the serologic assessment of pigs for virus infection. *Vet Microbiol* **123**, 1-14.

Labarque, G., Reeth, K. V., Nauwynck, H., Drexler, C., Van Gucht, S. & Pensaert, M. (2004). Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. *Vaccine* **22**, 4183-4190.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H. & Pensaert, M. (2003). Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. *Vet Microbiol* **95**, 187-197.

Labarque, G. G., Nauwynck, H. J., Van Reeth, K. & Pensaert, M. B. (2000). Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. *J Gen Virol* **81**, 1327-1334.

Lager, K. M. & Mengeling, W. L. (1995). Pathogenesis of in utero infection in porcine fetuses with porcine reproductive and respiratory syndrome virus. *Can J Vet Res* **59**, 187-192.

Martinez-Lobo, F. J., Diez-Fuertes, F., Simarro, I., Castro, J. M., & Prieto, C. (2011). Porcine reproductive and respiratory syndrome virus isolates differ in their susceptibility to neutralization. *Vaccine* **29**, 6928-6940.

Meier, W. A., Galeota, J., Osorio, F. A., Husmann, R. J., Schnitzlein, W. M. & Zuckermann, F. A. (2003). Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. *Virology* **309**, 18-31.

Mengeling, W. L., Lager, K. M., Wesley, R. D., Clouser, D. F., Vorwald, A. C. & Roof, M. B. (1999). Diagnostic implications of concurrent inoculation with attenuated and virulent strains of porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **60**, 119-122.

Mengeling, W. L., Lager, K. M. & Vorwald, A. C. (1994). Temporal characterization of transplacental infection of porcine fetuses with porcine reproductive and respiratory syndrome virus. *Am J Vet Res* **55**, 1391-1398.

Misinzo, G., Delputte, P. L., Meerts, P., Drexler, C. & Nauwynck, H. J. (2006). Efficacy of an inactivated PRRSV vaccine: induction of virus-neutralizing antibodies and partial virological protection upon challenge. *Adv Exp Med Biol* **581**, 449-454.

Molitor, T. W., Bautista, E. M. & Choi, C. S. (1997). Immunity to PRRSV: double-edged sword. *Vet Microbiol* **55**, 265-276.

Nelsen, C. J., Murtaugh, M. P. & Faaberg, K. S. (1999). Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol* **73**, 270-280.

Neumann, E. J., Kliebenstein, J. B., Johnson, C. D., Mabry, J. W., Bush, E. J., Seitzinger, A. H., Green, A. L. & Zimmerman, J. J. (2005). Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. *J Am Vet Med Assoc* **227**, 385-392.

Nielsen, H. S., Oleksiewicz, M. B., Forsberg, R., Stadejek, T., Bøtner, A. & Storgaard, T. (2001). Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J Gen Virol* **82**, 1263-1272.

Nielsen, T. L., Nielsen, J., Have, P., Baekbo, P., Hoff-Jorgensen, R. & Bøtner, A. (1997). Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **54**, 101-112.

Nilubol, D., Platt, K. B., Halbur, P. G., Torremorell, M. & Harris, D. L. (2004). The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. *Vet Microbiol* **102**, 11-18.

Okuda, Y., Kuroda, M., Ono, M., Chikata, S. & Shibata, I. (2008). Efficacy of vaccination with porcine reproductive and respiratory syndrome virus following challenges with field isolates in Japan. *J Vet Med Sci* **70**, 1017-1025.

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F. & Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology* **302**, 9-20.

Reed, L.J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493-497.

Tan, C., Chang, L., Shen, S., Liu, D. X. & Kwang, J. (2001). Comparison of the 5' leader sequences of North American isolates of reference and field strains of porcine reproductive and respiratory syndrome virus (PRRSV). *Virus Genes* **22**, 209-217.

Terpstra, C., Wensvoort, G. & Pol, J. M. (1991). Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet Q* **13**, 131-136.

Van Breedam, W., Costers, S., Vanhee, M., Geldhof, M. F., Verbeeck, M., Rodriguez Gomez, I., Van Doorsselaere, J., Gagnon, C. A. & Nauwynck, H. (2011). PRRSV-specific mAbs: supporting diagnostics and providing new insights into the antigenic properties of the virus. *Vet Immunol Immunopathol* **141**, 246-257.

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., Vandekerckhove, J., Van Damme, J., van Woensel, P. A. & Nauwynck, H. J. (2003). Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. *J Virol* **77**, 8207-8215.

Van Gorp, H., Van Breedam, W., Delputte, P. L. & Nauwynck, H. J. (2008). Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. *J Gen Virol* **89**, 2943-2953.

Vanhee, M., Van Breedam, W., Costers, S., Geldhof, M., Noppe, Y., & Nauwynck, H. J. (2011). Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use off peptide-specific serum antibodies. *Vaccine* **29**, 4794-4804.

Vanhee, M., Delputte, P. L., Delrue, I., Geldhof, M. F. & Nauwynck, H. J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Vet. Res* **40**, 63.

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. & et al. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q* **13**, 121-130.

Yoon, K. J., Zimmerman, J. J., Swenson, S. L., McGinley, M. J., Eernisse, K. A., Brevik, A., Rhinehart, L. L., Frey, M. L., Hill, H. T. & Platt, K. B. (1995). Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. *J Vet Diagn Invest* **7**, 305-312.

Zhou, Y. J., Yu, H., Tian, Z. J., Liu, J. X., An, T. Q., Peng, J. M., Li, G. X., Jiang, Y. F., Cai, X. H., Xue, Q., Wang, M., Wang, Y. F. & Tong, G. Z. (2009). Monoclonal antibodies and conserved antigenic epitopes in the C terminus of GP5 protein of the North American type porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **138**, 1-10.

Zuckermann, F. A., Garcia, E. A., Luque, I. D., Christopher-Hennings, J., Doster, A., Brito, M. & Osorio, F. (2007). Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol* **123**, 69-85.

CHAPTER 5

GENERAL DISCUSSION

5.1. INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) can cause reproductive failure in sows on itself, while PRRS-associated respiratory disease results from co-infections of PRRSV with other viral or bacterial pathogens (Brockmeier et al., 2002; Christianson et al., 1992; Terpstra et al., 1991).

To date, PRRSV has spread worldwide with the characteristics of an endemic in the swine-producing countries, causing enormous economic losses each year (Brouwer et al., 1994; Dea et al., 2000; Neumann et al., 2005). The high genetic and antigenic variability of PRRSV, underlying different phenotypic properties amongst virus strains, poses an important challenge for herd-level and regional control (Kapur et al., 1996; Kimman et al., 2009; Murtaugh et al., 1995; Nelsen et al., 1999). For example, PRRSV strains can largely differ in pathogenesis, virulence and interaction with the immune system (Darwich et al., 2011; Halbur & Bush, 1997; Tian et al., 2007).

Up till now, the most studied aspect of PRRSV immunity has been the virus-specific antibody response. Several studies specifically aimed to characterize the VN antibody response and its role in protection against PRRSV infection (Labarque et al., 2000; Lopez & Osorio, 2004; Lopez et al., 2007; Ostrowski et al., 2002; Yoon et al., 1995). Although resolution of PRRSV infection is not always directly correlated with the VN antibody response (Diaz et al., 2006), there is clear evidence that neutralizing antibodies can facilitate virus clearance and, when present in sufficient amounts, may even provide a sterilizing immunity (Labarque et al., 2000; Lopez & Osorio, 2004; Lopez et al., 2007; Osorio et al., 2002). While VN antibodies are certainly considered as an important element of protective immunity, it has become clear that also other immune components/mechanisms are necessary to provide efficient protection.

In addition to hygienic measures and management strategies, vaccination with inactivated (EU type) or attenuated (EU or NA type) vaccines is often applied in the field to prevent or control virus circulation. However, PRRSV keeps on circulating in the field, and even farms where strict vaccination procedures are applied may suffer from PRRS-associated problems (porcine veterinarians, personal communications; H. Nauwynck, personal communication; Thanawongnuwech & Suradhat, 2010). In a small study at pig farms in Flanders and The Netherlands, virus could be isolated from 16 out of 19 farms, independent of the vaccination protocols that were used on

the different farms (Geldhof, unpublished data). It is generally accepted that a continuous update of PRRSV vaccine strains is necessary to reach an acceptable level of protection in the field. However, the strains that are used in the commercial vaccines originate from the nineties. Our laboratory is doing a lot of efforts to design new vaccines based on new insights in the PRRSV infection pathogenesis and immune response (Nauwynck et al., 2012). Virus was inactivated by a quality-controlled viral inactivation procedure that optimally conserves the functional properties of the viral envelope proteins (Delrue et al., 2009). In addition, it was decided to vaccinate with high doses of inactivated virus and to administer the virus in combination with an oil-in-water adjuvant, normally used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky (Fort Dodge Animal Health, Kelmis, Belgium). Vaccination of naïve pigs with the experimental vaccine, based on Lelystad virus (LV), resulted in a strong LV-specific VN antibody response by vaccination on itself with a clear reduction of viremia upon homologous PRRSV challenge (Vanhee et al., 2009). Based on this background, a sufficient antigenic load and suitable adjuvant were used throughout this PhD work to induce an adequate immune response.

5.2. VACCINATION OF PRRSV-NAIVE ANIMALS UNDER HOMOLOGOUS AND HETEROLOGOUS EXPERIMENTAL CONDITIONS

Currently however, it is unknown whether it is possible to achieve similar results for PRRSV isolates that are currently causing reproductive or respiratory disorders in the field. In this thesis, recent PRRSV isolates, from outbreaks in herds vaccinated with a registered vaccine, were used for autogenous inactivated vaccine development. The capacity of inactivated PRRSV vaccines, grown on PK15$^{Sn\text{-}CD163}$ cells or on MARC-145 cells, to protect naïve pigs against homologous or heterologous PRRSV challenge was investigated. The induction of challenge virus-specific IPMA and VN antibody titers were examined, as well as the virological protection upon challenge - a factor directly linked with viral pathogenesis and spread. All farm-specific inactivated PRRSV vaccines were effective in partially protecting naïve pigs upon homologous challenge. They reduced the duration of viremia with 2 or more weeks upon homologous challenge, when compared to the viremic phase in the respective mock-vaccinated groups, which lasted roughly 1 month. Vaccination with BEI-inactivated, homologous MARC-145 grown virus resulted in a significant reduction of viremia

and a significant decrease in the number of viremic piglets upon challenge, while the heterologous BEI-inactivated, MARC-145 grown virus did not. Both homologous and heterologous BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown vaccines obtained a significant reduction of viremia and a significant decrease in the number of viremic piglets. These results are in line with an earlier study published by Vanhee et al. (2009), showing that BEI-inactivated vaccines can be successful in reducing viremia upon challenge. This is also the first report of an inactivated, $PK15^{Sn\text{-}CD163}$ grown PRRSV vaccine that manages to induce partial protection upon homologous and heterologous challenge. A possible explanation for the partial protection is that a high dose of virus was used for challenge (10^6 $TCID_{50}$) and that higher VN antibody titers may be needed at the time of challenge to offer full protection. Based on data by Benfield et al. (2000) the $TCID_{50}$ for field exposure via intranasal route is approximately 10^3-10^4 $TCID_{50}$.

Homologous and heterologous BEI-inactivated, MARC-145 grown as well as $PK15^{Sn\text{-}CD163}$ grown viruses induced a virus-specific antibody response. Vaccination with homologous and heterologous BEI-inactivated, MARC-145 grown virus consistently induced a VN antibody response upon booster vaccination. The homologous $PK15^{Sn\text{-}CD163}$ grown vaccine also induced VN antibodies upon double vaccination, with the induced titers being lower than the induced titers in MARC-145 grown, BEI-inactivated groups. In the pigs in the group vaccinated with heterologous, BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown virus the VN antibody response was only detectable upon challenge, but was strongly primed compared to the animals in the control group. The fast appearance of VN antibodies upon challenge for MARC-145 grown and for $PK15^{Sn\text{-}CD163}$ grown vaccines is in agreement with the findings in previous studies (Delrue, 2010; Misinzo et al., 2006; Vanhee et al., 2009). It demonstrates the potential of priming the neutralizing antibody response by immunization with a high dose of inactivated PRRSV. In addition, as described by other authors, (Labarque et al., 2003; Molitor et al., 1997; Vanhee et al., 2009) a correlation between the appearance of high VN antibody titers and the reduction of viremia was observed, indicating that VN antibodies may contribute to protection against the virus. Although it has been reported that the PRRSV-specific neutralizing antibody response is to a large extent strain specific and a lack in cross-neutralization may occur even between genetically closely related virus strains (Kim & Yoon, 2008; Okuda et al., 2008), our

data indicate that between the EU type viruses used in this thesis, cross-neutralization exists.

Although in the first study, (**chapter 3**) the used BEI-inactivated vaccines induced antibodies that could neutralize the homologous as well as the heterologous challenge virus in *in vitro* seroneutralization assays, it was somewhat surprising that these vaccines could only limit viremia under the homologous challenge conditions, and not when the heterologous challenge virus was used. The exact reason behind this remains currently unknown, but several possible explanations may be given. For instance, it is possible that induction of virus-specific neutralizing antibodies is not sufficient and that PRRSV vaccines must promote other immune mechanisms (e.g. via cross-presentation to T-cells) to provide a significant degree of protection upon challenge. On the other hand, it can be speculated that, although the vaccine-induced antibodies can bind and neutralize the homologous and heterologous challenge virus to a similar extent in *in vitro* SN assays, they recognize the homologous virus with a higher affinity. Affinity differences may explain a reduced binding and neutralization of heterologous virus *in vivo*, as the binding conditions for (VN) antibodies are likely more stringent *in vivo* than in the *in vitro* SN assays. Under homologous challenge conditions, the antibodies have undergone optimal challenge virus-specific affinity maturation, while this is not the case under heterologous challenge conditions. In theory, the presence of vaccine-induced antibodies that cross-react with a heterologous challenge virus may even prevent the selection of high-affinity (VN) antibodies against this challenge virus (original antigenic sin). Clearly, this matter requires further investigation in the future.

In the last study (**chapter 4**) the heterologous, BEI-inactivated, MARC-145 grown or $PK15^{Sn\text{-}CD163}$ grown vaccines induced antibodies that could neutralize the heterologous challenge virus in *in vitro* SN assays and consequently could limit viremia under heterologous challenge conditions. These results suggest that in PRRSV, under some circumstances heterologous VN antibodies can be equally efficient as the homologous VN antibodies. This was recently observed by Martinez-Lobo et al. (2011). Differences in cross-reactivity might be due to different reasons. A first factor is the antigenic variability of neutralizing epitopes (NE). Although different viral proteins, including GP2, GP3, GP4, GP5 and M protein, have been identified as inducers of VN antibodies by different approaches, the NEs of PRRSV

have not been fully characterized (Ansari et al., 2006; Cancel-Tirado et al., 2004; Kim & Yoon, 2008; Kim et al., 2007; Vanhee et al., 2011). Changes in the aa sequence of these NEs might prevent recognition of those epitopes by VN antibodies. Some hotspots of high variability are observed in the different proteins, while some other regions are strongly conserved, even between EU- and NA type strains. The level of aa conservation in a certain region always results from functional sequence restrictions that are required for the virus viability, together with immunological pressure that tends to select for mutant variants that escape immunity. Therefore, exceptionally conserved regions are thought to be highly functionally restricted, while extremely variable regions are thought to be subject to large immunological pressure (Costers et al., 2010a; Costers et al., 2010b; Vanhee et al., 2011). For example, the highly variable region in GP4 is known to induce VN antibodies for LV and for field isolates. However, the large variability of this region makes that GP4-specific neutralizing antibodies are poorly cross-protective between different virus strains (Vanhee et al., 2010).

A second factor that can change the VN profile is glycosylation of envelope proteins that is used by enveloped viruses as a mechanism of immune evasion used to escape, block or minimize virus VN antibody response making epitopes poorly immunogenic (Ansari et al., 2006). This strategy has been demonstrated for several viruses, including human immunodeficiency virus 1 (Binley et al., 2010), hepatitis B virus (Lee et al., 2003) and hepatitis C virus (Gal-Tanamy et al., 2008). Still, the nature of this phenomenon remains currently unknown. Nevertheless, in the study by Martínez-Lobo et al. (2011) differences in cross-neutralization in sera raised against different EU type strains could not be related strictly to the sequence and number of glycosylations of the known GP3, GP4 or GP5 NE and those authors suggested that maybe the conformational characteristics of the epitopes could have a role on the cross-reactivity or, alternatively, that other NE unknown yet exist.

In general, adaptive immunity induced by a certain virus strain is often not (fully) cross-protective against other strains (Kim & Yoon, 2008; Labarque et al., 2004; Mengeling et al., 2003). Still, the relative contribution of different immune determinants in protection is not fully elucidated, and no straightforward clue exists to date to accurately predict cross-reactivity. Clearly, this matter requires further investigation in the future.

5.3. CELL LINES: MARC-145 GROWN OR PK15$^{Sn\text{-}CD163}$ GROWN

A limited number of options for vaccine production are available to grow PRRSV *in vitro*. *In vivo*, PRRSV infects a subpopulation of differentiated macrophages (Duan et al., 1998). Besides these primary porcine cells (PAM), only the African green monkey kidney cells MA-104, and cells derived thereof, such as MARC-145, sustain *in vitro* virus replication (Kim et al., 1993). These MARC-145 cells however lack sialoadhesin (Sn) and as a consequence the virus is forced to enter MARC-145 cells exclusively in a Sn-independent way, which is notably different from entry in the PRRSV in vivo target cells, macrophages, where the main entry pathway is Sn-dependent. This forced shift in entry-pathway may be one of the reasons that adaptation is needed for PRRSV to grow on MARC-145 cells (Collins et al., 1992; Tan et al., 2001). Due to adaptation, mutations in non-structural, but also structural viral proteins can occur (Allende et al., 2000; Indik et al., 2000; Zhou et al., 2009), which can lead to an inefficient immune response. Upon discovery of CD163 as a key component of PRRSV entry, non-permissive cells expressing CD163 were proposed as alternative to PAM and MARC-145, due to their potential to sustain productive PRRSV infection (Calvert et al., 2007). Since the main entry pathway of PRRSV in macrophages involves Sn-mediated internalization in addition to CD163, a non-permissive cell line expressing both sialoadhesin and CD163 was developed in our laboratory. Preliminary results in our laboratory showed that primary PRRSV isolates are indeed able to grow immediately without passaging on these CD163^{+}SN^{+} PK15 cells, albeit not all of them, a phenomenon that is also observed for macrophages (de Abin et al., 2009). Additionally, virus yield was significantly higher on CD163^{+}SN^{+} PK15 cells compared to cells solely expressing CD163 (Van Gorp Hanne, personal communication). Furthermore, the virus yield was equal or even higher than on PAM or MARC-145 cells (Delrue et al., 2010). The production of different PRRSV strains on those cell lines was optimized. In this thesis, the capacity of inactivated PRRSV vaccines, grown on PK15$^{Sn\text{-}CD163}$ cells or on MARC-145 cells, to protect naïve pigs against homologous or heterologous PRRSV challenge was examined. For both PRRSV isolates used in the study, it was shown by sequencing that mutation of structural viral proteins did not occur after passaging in PK15$^{Sn\text{-}CD163}$ cells. It is important that no mutations occur in the ORFs encoding the structural viral proteins, since these are important for the induction of a VN antibody response and protection

against infection. Though, mutations in ORFs encoding structural viral proteins after growth on PK15$^{Sn\text{-}CD163}$ cells cannot always be avoided for all PRRSV strains (Delrue et al., 2010). Considering the similar efficacy in homologous situations of MARC-145 grown or PK15$^{Sn\text{-}CD163}$ grown vaccines virus, it can be questioned whether the use of PK15$^{Sn\text{-}CD163}$ grown vaccines is advantageous over the use of MARC-145 grown vaccines. Hurdles concerning MARC-145 cells are the difficulties to adapt PRRSV to MARC-145 cells (Collins et al., 1992; Tan et al., 2001) and patents that restrict the commercial use of these cells. Most field isolates do not replicate in MARC-145 cells, with a report showing that only 2% of the tested field samples replicated in MARC-145 cells, contrasting to 70% in PAM (de Abin et al., 2009). Recent preliminary results in our laboratory showed that primary PRRSV field isolates are able to grow immediately without passaging in PK15$^{Sn\text{-}CD163}$ cells, albeit not all of them. Further research is needed to explore the value of this promising Sn-positive PK15 cell line stably expressing CD163, thereby focussing on virus isolation and production, and its potential as a tool for in vitro PRRSV studies.

5.4. COMMERCIAL AVAILABLE VACCINES

Separate from the experimental BEI-inactivated PRRSV vaccines, also 1 commercial inactivated (Progressis®) and 2 commercial attenuated (Porcilis® PRRS and Ingelvac® PRRS MLV) PRRSV vaccines were included in the first study and served as a reference. Vaccination with the commercial inactivated vaccine Progressis® did not induce VN antibodies, neither did it provide any degree of protection in naïve pigs upon challenge. This is in line with the results from previous studies, showing that the commercial inactivated vaccines do not influence viremia, even in nearly homologous conditions (Nielsen et al., 1997; Scortti et al., 2007; Zuckermann et al., 2007). Vaccination with the EU genotype attenuated vaccine reduced the duration of viremia upon challenge with 07V063 with approximately one week. In animals challenged with 08V194, this vaccine reduced viremia from 5 to 2 weeks. The NA genotype, attenuated vaccine reduced viremia in 07V063-challenged animals with approximately one week. Hence, despite the concerns regarding the efficacy of the attenuated vaccine used on both farms, the results from our study indicate that the use of this vaccine in PRRS-naïve pigs can clearly limit viremia. These results are in line with earlier studies published by Cano et al. (2007) and Scortti et al. (2006), showing

that attenuated vaccines can be successful in controlling and reducing clinical disease upon homologous and heterologous challenge.

In the animals vaccinated with the commercial inactivated PRRSV vaccine Progressis®, neither the IPMA nor the VN antibody response was influenced before or after challenge with 07V063, which is in line with the results reported by Zuckermann et al. (2007) and Vanhee et al. (2009), where they used the same vaccine and the LV strain as challenge virus: no clear induction of challenge virus-specific (neutralizing) antibodies was observed upon vaccination with the commercial inactivated PRRSV vaccine and only a moderate anamnestic antibody response was observed upon challenge of the vaccinated animals. The apparent limited immunogenicity of this vaccine may relate to the inactivation procedure used, strain variability, antigenic dose, adjuvant, … Further research is necessary to elucidate this. In the animals vaccinated with the commercial attenuated vaccines, either based on EU- or NA type virus, a low or non-detectable VN antibody response was observed, which is in agreement with the results of Lopez & Osorio (2004). None of the attenuated vaccines were able to induce a faster neutralizing antibody response upon challenge. Despite the absence of a clear challenge virus-specific VN antibody response, the commercial attenuated vaccines do provide a partial virological protection, roughly similar to the protection provided by the autogenous BEI-inactivated vaccines. This observation points towards a significant role of other attenuated vaccine-induced immune mechanisms (e.g. cell-mediated immunity) in the protection against PRRSV infection (Charerntantanakul et al., 2006; Diaz et al., 2006; Zuckermann et al., 2007).

5.5. VACCINATION OF PREGNANT PRRSV-NAIVE GILTS UNDER EXPERIMENTAL CONDITIONS

Preventing the virus spread from mother to fetus is an important step towards control of PRRSV-related reproductive problems (Plana et al., 1992; Terpstra et al., 1991). The exact mechanism of the virus-induced reproductive failure remains unknown. Recent findings have shown that PRRSV efficiently replicates in the fetal implantation sites (endometrium/fetal placenta) during late gestation and causes apoptosis in infected and surrounding cells (Karniychuk et al., 2011). Therefore, the BEI-inactivated vaccine was tested in pregnant naïve gilts (Karniychuk et al., 2012). The experimental inactivated PRRSV vaccine primed the VN antibody response and

slightly reduced the duration of vriremia in gilts. Furthermore, vaccination reduced the virus replication in the fetal placenta and placental pathology and lowered the number of virus-positive fetuses. Vaccine-mediated factors are supposed to be the reason for reducing PRRSV transfer from the endometrium (the primary site for PRRSV replication prior to conceptus infection) to the fetal placenta. However, congenital infection could not fully be prevented. Previously, other inactivated vaccines were neither able to prevent congenital infection (Plana-Duran et al., 1997; Scortti et al., 2007). In the study of Karniychuk et al. (2012), a protocol for primary testing of PRRSV vaccines in pregnant animals was proposed that might be useful for testing vaccines against PRRSV in pregnant sows in a standardized way.

5.6. VACCINATION OF NON-PREGNANT PRRSV-IMMUNE SOWS UNDER EXPERIMENTAL CONDITIONS AND PREGNANT PRRSV-IMMUNE SOWS UNDER FIELD CONDITIONS

Despite the frequent use of commercially available vaccines for maintaining immunity in breeding herds, little is known on how the used vaccines boost the antibody response against divergent virus strains. The presence of sufficient amounts of VN antibodies can fully prevent the transplacental infection with PRRSV and completely extinguish the infection of PRRSV in pregnant females (Osorio et al., 2002). These findings are in agreement with previous reports of commercial or experimental vaccines inducing VN antibodies, which seemed to be associated with protection (Osorio et al., 1998; Pirzadeh and Dea, 1998). These data support the interesting possibility that passive transfer of VN antibodies via colostrum could be used to provide instant protection of animals at high risk of infection with PRRSV. This thesis investigated the serological response of non-pregnant sows, which came from herds with active circulation of naturally occurring PRRSV-variants, upon vaccination with commonly used commercial vaccines (one inactivated and two attenuated vaccines) or a farm-specific BEI-inactivated vaccine.

Vaccination with the commercial attenuated vaccines elicited a variable antibody response. In contrast, vaccination with the attenuated NA-type vaccine (Ingelvac® PRRS) did not elicit virus-specific or VN antibody production against the PRRSV-variants. These results support the idea that the current generation of attenuated vaccines is not universally successful in stimulating the (heterologous) humoral immune response in infection-immune animals. This is in line with a study by

Murtaugh et al. (2002), who reported that attenuated vaccines have difficulties in maintaining immune protection in sows. The absence of an anamnestic humoral immune response upon re-vaccination may result from a lack in sufficient vaccine virus replication in vaccinated animals, although strain differences between the vaccine and challenge virus may also explain this phenomenon (Charerntantanakul et al., 2006; Scortti et al., 2006; Zuckermann et al., 2007). The inactivated EU-type vaccine (Progressis®) stimulated the virus-specific antibody response and also a moderate anamnestic VN antibody response against 2 different genetically different PRRSV strains. Although the commercial inactivated vaccines do not appear to stimulate the VN antibody response in naïve animals (Vanhee et al., 2009; Zuckermann et al., 2007), it has been reported that they may boost VN antibody production in previously infected animals (Meier et al., 2003; Nilubol et al., 2004; Plana-Duran et al., 1997; Scortti et al., 2007; Zimmerman et al., 2006). All sows vaccinated with the farm-specific inactivated vaccines, showed a significant rise in VN antibodies against the prevalent PRRSV-variant in their herd. In previous studies, a correlation was seen between the induction of VN antibodies by this kind of BEI-inactivated adaptable PRRSV vaccine and partial protection upon homologous challenge in naïve animals (Geldhof et al., 2012; Vanhee et al., 2009). This boosting of sows with the BEI-inactivated farm-specific vaccines results in the rise of VN antibodies in the blood and may lead to protection against transplacental spread and birth of viremic piglets (Lopez et al., 2007; Osorio et al., 2002). The impact of a BEI-inactivated autogenous vaccine on maternal immunity and on the PRRSV infection pattern in piglets during their first weeks on a farm was investigated in this thesis. High levels of VN antibodies were detected in serum of farm-specific immunized sows at 2 weeks upon vaccination. The serological response after vaccination with Porcilis® PRRS varied between sows. The concentration of antibodies in sow serum was shown to decline the first week post-partum and remained low, similar to pre-partum levels until the end of lactation. A high amount of maternal VN antibodies in serum was found at 3 and 5 weeks after birth in 100% and 81% of the piglets that ingested colostrum from sows vaccinated with the farm-specific vaccine. In contrast, the VN antibody titers of piglets from the Porcilis® PRRS -vaccinated sows decreased faster (32% positive at 5 weeks) and only a few piglets of 3 mock-vaccinated sows had VN antibody titers against the circulating farm-specific isolate supporting that the sow population experienced a contact with the circulating PRRSV-variant during their

life. This also resulted in a faster appearance of viremic piglets and a statistical higher number of viremic piglets in the piglets of the non-vaccinated sows at 7 and 9 weeks of age. The appearance of viremic piglets of vaccinated (both farm-specific and Porcilis® PRRS) sows was delayed in time in comparison with piglets of control sows. The passive transfer of VN antibodies in colostrum to piglets has lead to a curtailment of viremic piglets. When colostral VN antibodies became undetectable, the number of viremic piglets increased. Lopez et al. (2007) has observed that passively transferred VN antibodies protect young piglets against PRRSV infection. Neutralizing antibodies can block viremia, as described before in reports by Yoon et al. (1996) and Labarque et al. (2003). Despite a lower transfer of VN antibody titers from sow to piglets in Porcilis® PRRS vaccinated sows, results from our study indicated that the use of this vaccine in sows still has positive effects in the nursery. As previously documented, not only antibody immunity, but also other immune factors could be transferred by colostrum (Bandrick et al., 2008). These colostrum-derived immune factors may as well have played a role in the immunity of these piglets. Similar results as above-mentioned were observed on a second farm (data not shown). High titers of maternal VN antibodies in serum were found at 3, 5 and 7 weeks after birth in 100%, 81% and 67% of the piglets that ingested colostrum from sows vaccinated with this farm-specific vaccine. Remarkably, at 3 weeks of age, 67% of the piglets of mock-vaccinated sows had VN antibody titers against the circulating farm-specific isolate. In total, one viremic piglet was detected at 9 weeks of age, suggesting that the piglets up to 8 weeks of age are well protected against PRRSV circulation. High VN antibody titers were present in all sows, at the time of the vaccination, suggesting that the sows of this farm had a strong population-immunity (due to vaccination or maybe a recent infection). Large-scale use of attenuated vaccines can stimulate the immunity in infectious-immune animals and thus reduce negative consequences of heterologous PRRSV strains.

Data obtained in our study should help us to understand the importance of maternal vaccination in order to protect the offspring from PRRSV-infections. A more elaborated exploration on this topic should be done in the future.

5.7. OTHER IMMUNE MECHANISMS

Although there is a correlation between the appearance of VN antibodies and the elimination of PRRSV from the circulation (Batista et al., 2004; Labarque et al., 2000), it should not be forgotten that VN antibodies are not the sole players in PRRSV-specific protective immunity (Charerntantanakul et al., 2006; Diaz et al., 2006; Zuckermann et al., 2007). Due to the absence of a strong VN antibody response, the efficacy of attenuated vaccines points towards a significant role of other effector mechanisms in the final protection against PRRSV (Charerntantanakul et al., 2006; Diaz et al., 2006; Zuckermann et al., 2007). PRRSV-specific cell-mediated immunity also develops very gradually upon infection in naïve animals and it is currently not clear how and to which extent cellular (vaccine-induced) immune mechanisms contribute to protection and which cell types are involved (Charerntantanakul et al., 2006; Costers et al., 2009; Diaz et al., 2005; Meier et al., 2003). Nevertheless, animals that have gone through a PRRSV infection are generally well protected against a second infection with the homologous virus strains, and both antibody-dependent as well as cell-mediated immune components are supposed to be involved in this protective immunity (Lager et al., 1999; Meier et al., 2003; Mengeling et al., 2003; Osorio et al., 2002).

REFERENCES

Allende, R., Laegreid, W. W., Kutish, G. F., Galeota, J. A., Wills, R. W. & Osorio, F. A. (2000). Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. *J Virol* **74**, 10834-10837.

Ansari, I. H., Kwon, B., Osorio, F. A. & Pattnaik, A. K. (2006). Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol* **80**, 3994-4004.

Bandrick, M., Pieters, M., Pijoan, C. & Molitor, T.W. (2008). Passive transfer of maternal Mycoplasma hyopneumoniae-specific cellular immunity to piglets. *Clin Vaccine Immunol* **15**, 540-543.

Batista, L., Pijoan, C., Dee, S., Olin, M., Molitor, T., Joo, H. S., Xiao, Z. & Murtaugh, M. (2004). Virological and immunological responses to porcine reproductive and respiratory syndrome virus in a large population of gilts. *Can J Vet Res* **68**, 267-273.

Benfield, D. A., Nelson, C., Steffen, M. & Rowland, R. R. R. (2000). Transmission of PRRSV by artificial insemination using extended semen seeded with differnet concentrations of PRRSV. *Proc Annu Meet Am Assoc Swine pract* 405-408.

Binley, J. M., Ban, Y. E., Crooks, E. T., Eggink, D., Osawa, K., Schief, W. R. & Sanders, R. W. (2010). Role of complex carbohydrates in human immunodeficiency virus type 1 infection and resistance to antibody neutralization. *J Virol* **84**, 5637-5655.

Brockmeier, S. L., Halbur, P. & Thacker, E. (2002). Porcine Respiratory Disease Complex. In *Polymicrobial Diseases*. Edited by K. A. Brogden & J. M. Guthmiller. Washington (DC): ASM Press.

Brouwer, J., Frankena, K., de Jong, M. F., Voets, R., Dijkhuizen, A., Verheijden, J. & Komijn, R. E. (1994). PRRS: effect on herd performance after initial infection and risk analysis. *Vet Q* **16**, 95-100.

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G. & Welch, S. K. (2007). CD163 Expression Confers Susceptibility to Porcine Reproductive and Respiratory Syndrome Viruses. *J Virol* **81**, 7371-7379.

Cancel-Tirado, S. M., Evans, R. B. & Yoon, K. J. (2004). Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. *Vet Immunol Immunopathol* **102**, 249-262.

Cano, J. P., Dee, S. A., Murtaugh, M. P. & Pijoan, C. (2007a). Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. *Vaccine* **25**, 4382-4391.

Charerntantanakul, W., Platt, R., Johnson, W., Roof, M., Vaughn, E. & Roth, J. A. (2006). Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol* **109**, 99-115.

Christianson, W. T., Collins, J. E., Benfield, D. A., Harris, L., Gorcyca, D. E., Chladek, D. W., Morrison, R. B. & Joo, H. S. (1992). Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. *Am J Vet Res* **53**, 485-488.

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., Goyal, S. M., McCullough, S., Morrison, R. B., Joo, H. S. & et al. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest* **4**, 117-126.

Costers, S., Lefebvre, D. J., Van Doorsselaere, J., Vanhee, M., Delputte, P. L. & Nauwynck, H. J. (2010a). GP4 of porcine reproductive and respiratory syndrome virus contains a neutralizing epitope that is susceptible to immunoselection in vitro. *Arch Virol* **155**, 371-378.

Costers, S., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Geldhof, M. & Nauwynck, H. J. (2010b). GP4-specific neutralizing antibodies might be a driving force in PRRSV evolution. *Virus Res* **154**, 104-113.

Costers, S., Lefebvre, D. J., Goddeeris, B., Delputte, P. L. & Nauwynck, H. J. (2009). Functional impairment of PRRSV-specific peripheral CD3+CD8high cells. *Vet Res* **40**, 46.

Darwich, L., Gimeno, M., Sibila, M., Diaz, I., de la Torre, E., Dotti, S., Kuzemtseva, L., Martin, M., Pujols, J. & Mateu, E. (2011). Genetic and immunobiological diversities of porcine reproductive and respiratory syndrome genotype I strains. *Vet Microbiol* **150**, 49-62.

Dea, S., Gagnon, C. A., Mardassi, H., Pirzadeh, B. & Rogan, D. (2000). Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. *Arch Virol* **145**, 659-688.

de Abin, M. F., Spronk, G., Wagner, M., Fitzsimmons, M., Abrahante, J. E. & Murtaugh, M. P. (2009). Comparative infection efficiency of porcine reproductive and respiratory syndrome virus field isolates on MA104 cells and porcine alveolar macrophages. *Can J Vet Res* **73**, 200-204.

Delrue, I., Van Gorp, H., Van Doorsselaere, J., Delputte, P. L. & Nauwynck, H. J. (2010). Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol* **10**, 48.

Delrue, I., Delputte, P. L. & Nauwynck, H. J. (2009). Assessing the functionality of viral entry-associated domains of porcine reproductive and respiratory syndrome virus during inactivation procedures, a potential tool to optimize inactivated vaccines. *Vet Res* **40**, 62.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. (2006). Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. *Virology* **351**, 249-259.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. & Mateu, E. (2005). Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. *J Gen Virol* **86**, 1943-1951.

Duan, X., Nauwynck, H. J., Favoreel, H. W. & Pensaert, M. B. (1998). Identification of a putative receptor for porcine reproductive and respiratory syndrome virus on porcine alveolar macrophages. *J Virol* **72**, 4520-4523.

Gal-Tanamy, M., Keck, Z. Y., Yi, M., McKeating, J. A., Patel, A. H., Foung, S. K. & Lemon, S. M. (2008). In vitro selection of a neutralization-resistant hepatitis C virus escape mutant. *Proc Natl Acad Sci USA* **105**, 19450-19455.

Geldhof, M. F., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Karniychuk, U. & Nauwynck, H. J. (2012). Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. *BMC Vet Res* **8**, 182.

Halbur, P. G. & Bush, E. (1997). Update on abortion storms and sow mortality. *Swine Health and Production* **5**, 73-73.

Indik, S., Valicek, L., Klein, D. & Klanova, J. (2000). Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **81**, 2497-2502.

Kapur, V., Elam, M. R., Pawlovich, T. M. & Murtaugh, M. P. (1996). Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. *J Gen Virol* **77**, 1271-1276.

Karniychuk, U. U., Saha, D., Vanhee, M., Geldhof, M. F., Cornillie, P., Caij, A. B., De Regge, N. & Nauwynck, H. J. (2012). Impact of a novel inactivated PRRSV vaccine on virus replication and virus-induced pathology in fetal implantation sites and foetuses upon challenge. *Theriogenology* **78**, 1527-1537.

Karniychuk, U. U., Saha, D., Geldhof, M., Vanhee, M., Cornillie, P., Van den Broeck, W. & Nauwynck, H. J. (2011). Porcine reproductive and respiratory syndrome virus (PRRSV) causes apoptosis during its replication in fetal implantation sites *Microb Pathog* **51**, 194-202.

Kim, W. I. & Yoon, K. J. (2008). Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. *Virus Genes* **37**, 380-391.

Kim, W. I., Lee, D. S., Johnson, W., Roof, M., Cha, S. H. & Yoon, K. J. (2007). Effect of genotypic and biotypic differences among PRRS viruses on the serologic assessment of pigs for virus infection. *Vet Microbiol* **123**, 1-14.

Kim, H. S., Kwang, J., Yoon, I. J., Joo, H. S. & Frey, M. L. (1993). Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch Virol* **133**, 477-483.

Kimman, T. G., Cornelissen, L. A., Moormann, R. J., Rebel, J. M. & Stockhofe-Zurwieden, N. (2009). Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. *Vaccine* **27**, 3704-3718.

Labarque, G., Reeth, K. V., Nauwynck, H., Drexler, C., Van Gucht, S. & Pensaert, M. (2004). Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. *Vaccine* **22**, 4183-4190.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H. & Pensaert, M. (2003). Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. *Vet Microbiol* **95**, 187-197.

Labarque, G. G., Nauwynck, H. J., Van Reeth, K. & Pensaert, M. B. (2000). Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. *J Gen Virol* **81**, 1327-1334.

Lager, K. M., Mengeling, W. L. & Brockmeier, S. L. (1999). Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. *Am J Vet Res* **60**, 1022-1027.

Lee, J., Park, J. S., Moon, J. Y., Kim, K. Y. & Moon, H. M. (2003). The influence of glycosylation on secretion, stability, and immunogenicity of recombinant HBV pre-S antigen synthesized in Saccharomyces cerevisiae. *Biochem Biophys Res Commun* **303**, 427-432.

Lopez, O. J., Oliveira, M. F., Garcia, E. A., Kwon, B. J., Doster, A. & Osorio, F. A. (2007). Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. *Clin Vaccine Immunol* **14**, 269-275.

Lopez, O. J. & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol* **102**, 155-163.

Martinez-Lobo, F. J., Diez-Fuertes, F., Simarro, I., Castro, J. M., & Prieto, C. (2011). Porcine reproductive and respiratory syndrome virus isolates differ in their susceptibility to neutralization. *Vaccine* **29**, 6928-6940.

Meier, W. A., Galeota, J., Osorio, F. A., Husmann, R. J., Schnitzlein, W. M. & Zuckermann, F. A. (2003). Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. *Virology* **309**, 18-31.

Mengeling, W. L., Lager, K. M., Vorwald, A. C. & Koehler, K. J. (2003). Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **93**, 13-24.

Misinzo, G., Delputte, P. L., Meerts, P., Drexler, C. & Nauwynck, H. J. (2006). Efficacy of an inactivated PRRSV vaccine: induction of virus-neutralizing antibodies and partial virological protection upon challenge. *Adv Exp Med Biol* **581**, 449-454.

Molitor, T. W., Bautista, E. M. & Choi, C. S. (1997). Immunity to PRRSV: double-edged sword. *Vet Microbiol* **55**, 265-276.

Murtaugh, M. P., Xiao, Z. & Zuckermann, F. (2002). Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol* **15**, 533-547.

Murtaugh, M. P., Elam, M. R. & Kakach, L. T. (1995). Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Arch Virol* **140**, 1451-1460.

Nauwynck, H. J., Van Gorp, H., Vanhee, M., Karniychuk, U., Geldhof, M., J., Verbeeck, M. & Van Breedam, W. (2012). Micro-Dissecting the Pathogenesis and Immune Response of PRRSV Infection Paves the Way for More Efficient PRRSV Vaccines. *Transbound Emerg Dis* doi: 10.1111/j.1865-1682.2011.01292.x.

Nelsen, C. J., Murtaugh, M. P. & Faaberg, K. S. (1999). Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol* **73**, 270-280.

Neumann, E. J., Kliebenstein, J. B., Johnson, C. D., Mabry, J. W., Bush, E. J., Seitzinger, A. H., Green, A. L. & Zimmerman, J. J. (2005). Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. *J Am Vet Med Assoc* **227**, 385-392.

Nielsen, T. L., Nielsen, J., Have, P., Baekbo, P., Hoff-Jorgensen, R. & Bøtner, A. (1997). Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **54**, 101-112.

Nilubol, D., Platt, K. B., Halbur, P. G., Torremorell, M. & Harris, D. L. (2004). The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. *Vet Microbiol* **102**, 11-18.

Okuda, Y., Kuroda, M., Ono, M., Chikata, S. & Shibata, I. (2008). Efficacy of vaccination with porcine reproductive and respiratory syndrome virus following challenges with field isolates in Japan. *J Vet Med Sci* **70**, 1017-1025.

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F. & Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology* **302**, 9-20.

Osorio, F. A., Zuckermann, F., Wills, R., Meier, W., Christian, S., Galeota, S. &Doster, A. (1998). PRRSV: comparison of commercial vaccines in their ability to induce protection against current PRRSV strains of high virulence. In: *Leman, D.A. (Ed.) Swine Conf.* **25**, 176–182.

Ostrowski, M., Galeota, J. A., Jar, A. M., Platt, K. B., Osorio, F. A. & Lopez, O. J. (2002). Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol* **76**, 4241-4250.

Pirzadeh, B. & Dea, S. (1998). Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **79**, 989-999.

Plana, J., Vayreda, M., Vilarrasa, J., Bastons, M., Rosell, R., Martinez, M., Gabriel, A. S., Pujols, J., Badiola, J. L., Ramos, J. A. & Domingo, M. (1992). Porcine Epidemic Abortion and Respiratory Syndrome (Mystery Swine Disease) - Isolation in Spain of the Causative Agent and Experimental Reproduction of the Disease. *Vet Microbiol* **33**, 203-211.

Plana-Duran, J., Bastons, M., Urniza, A., Vayreda, M., Vila, X. & Mane, H. (1997). Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **55**, 361-370.

Scortti, M., Prieto, C., Alvarez, E., Simarro, I. & Castro, J. M. (2007). Failure of an inactivated vaccine against porcine reproductive and respiratory syndrome to protect gilts against a heterologous challenge with PRRSV. *Vet Rec* **161**, 809-813.

Scortti, M., Prieto, C., Simarro, I. & Castro, J. M. (2006). Reproductive performance of gilts following vaccination and subsequent heterologous challenge with European strains of porcine reproductive and respiratory syndrome virus. *Theriogenology* **66**, 1884-1893.

Tan, C., Chang, L., Shen, S., Liu, D. X. & Kwang, J. (2001). Comparison of the 5' leader sequences of North American isolates of reference and field strains of porcine reproductive and respiratory syndrome virus (PRRSV). *Virus Genes* **22**, 209-217.

Terpstra, C., Wensvoort, G. & Pol, J. M. (1991). Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet Q* **13**, 131-136.

Thanawongnuwech, R. & Suradhat, S. (2010). Taming PRRSV: Revisiting the control strategies and vaccine design. *Virus* **154**, 133-140.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J. & Gao, G. F. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS ONE* **2**, e526.

Vanhee, M., Van Breedam, W., Costers, S., Geldhof, M., Noppe, Y. & Nauwynck, H. J. (2011). Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use off peptide-specific serum antibodies. *Vaccine* **29**, 4794-4804.

Vanhee, M., Costers, S., Van Breedam, W., Geldhof, M., Van Doorsselaere, J. & Nauwynck, H. J. (2010). A variable region in GP4 of European-type porcine reproductive and respiratory syndrome virus induces neutralizing antibodies against homologous but not heterologous virus strains. *Viral Immunol* **23**, 403-413.

Vanhee, M., Delputte, P. L., Delrue, I. & Nauwynck, H. J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Vet. Res* **40**, 63.

Yoon, K. J., Wu, L. L., Zimmerman, J. J., Hill, H. T. & Platt, K. B. (1996). Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. *Viral Immunol* **9**, 51-63.

Yoon, K. J., Zimmerman, J. J., Swenson, S. L., McGinley, M. J., Eernisse, K. A., Brevik, A., Rhinehart, L. L., Frey, M. L., Hill, H. T. & Platt, K. B. (1995). Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. *J Vet Diagn Invest* **7**, 305-312.

Zhou, Y. J., Yu, H., Tian, Z. J., Liu, J. X., An, T. Q., Peng, J. M., Li, G. X., Jiang, Y. F., Cai, X. H., Xue, Q., Wang, M., Wang, Y. F. & Tong, G. Z. (2009). Monoclonal antibodies and conserved antigenic epitopes in the C terminus of GP5 protein of the North American type porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **138**, 1-10.

Zimmerman, J., Benfield, D. A., Murtaugh, M. P., Osorio, F., Stevenson, G. W. & Torremorell, M. (2006). Porcine Reproductive and Respiratory Syndrome Virus (Porcine Arterivirus). In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J., (Eds.), Diseases of Swine 9th Edition. Blackwell Science, USA, pp. 387-417.

Zuckermann, F. A., Garcia, E. A., Luque, I. D., Christopher-Hennings, J., Doster, A., Brito, M. & Osorio, F. (2007). Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol* **123**, 69-85.

CHAPTER 6

SUMMARY / SAMENVATTING

SUMMARY

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus that is involved with reproductive failure (weak- and stillborn piglets, premature farrowing, late-term abortions) in sows and respiratory disease in pigs of all ages, resulting in huge economic losses to the swine industry. A high genetic variability has been demonstrated within PRRSV variants and the genetic differences between virus variants are mirrored in different virulence, pathogenicity, immunogenicity, … This high variability of the virus represents a major hurdle for effective PRRSV prevention and control. Broad application of current available PRRSV vaccines is the most commonly used strategy to combat the clinical and economical impact of PRRSV infections. It is generally accepted that there is a need for new and safe vaccines that can protect against infection with those virus variants that escape immunity induced by the currently available commercial vaccines. The general goal of this work was to evaluate autogenous inactivated PRRSV vaccines – prepared according to a previously optimized in-house protocol – with the capacity of commercially available attenuated/inactivated PRRSV vaccines in naïve and PRRSV-immune animals. Currently however, it is unknown whether added benefits with these new method farm-specific vaccines can be made.

Chapter 1 shortly highlights the current literature on general aspects of the virus and the disease, PRRSV-specific immunity and PRRSV vaccines.

In **chapter 2** the general aim of this thesis is described and a number of aims are stated. The next 2 chapters consist of experimental data addressing these aims.

The first study in this thesis (**chapter 3 part 1)** mainly focused on the efficacy of the experimental BEI-inactivated vaccines against homologous and heterologous challenge and to compare it with an experimental LV-based BEI-inactivated vaccine and commercial inactivated and attenuated vaccines. In addition, the induction of challenge virus-specific (neutralizing) antibodies by the different vaccines was assessed. Two recent PRRSV field isolates (07V063 and 08V194) were used for BEI-inactivated vaccine production. In a first experiment (challenge with 07V063), vaccination with the experimental homologous (07V063) inactivated vaccine shortened the viremic phase upon challenge with approximately 2 weeks compared to the mock-vaccinated control group. Vaccination with the commercial attenuated

vaccines reduced the duration of viremia with approximately one week compared to the mock-vaccinated control group. In contrast, the experimental heterologous (LV) inactivated vaccine and the commercial inactivated vaccine did not influence viremia. Interestingly, both the homologous and the heterologous experimental inactivated vaccine induced 07V063-specific neutralizing antibodies upon vaccination, while the commercial inactivated and attenuated vaccines failed to do so. In the second experiment (challenge with 08V194), use of the experimental homologous (08V194) inactivated vaccine shortened viremia upon challenge with approximately 3 weeks compared to the mock-vaccinated control group. Similar results were obtained with the commercial attenuated vaccine. The experimental heterologous (07V063 and LV) inactivated vaccines did not significantly alter viremia. In this experiment, the experimental homologous and heterologous inactivated vaccines induced 08V194-specific neutralizing antibodies and a faster appearance post challenge was observed with the commercial attenuated vaccine. The main conclusions obtained from this study are that the experimental homologous inactivated vaccines significantly shortened viremia upon challenge. Despite the concerns regarding the efficacy of the commercial attenuated vaccines used on the farms where the field isolates were obtained, use of commercial attenuated vaccines clearly shortened the viremic phase upon challenge. In contrast, the experimental heterologous inactivated vaccines and the commercial inactivated vaccine had no or only a limited influence on viremia.

The study in **chapter 3 part 2** aimed to assess the capacity of commercially available attenuated/inactivated PRRSV vaccines and autogenous BEI-inactivated PRRSV vaccines to boost the antibody immunity against currently circulating PRRSV variants in PRRSV-immune sows. PRRSV isolates (07V063, 08V194 and 08V204) were obtained from 3 different swine herds experiencing PRRSV-related problems, despite regular vaccination of gilts and sows against the virus. In a first part of the study, the PRRSV-specific antibody response upon booster vaccination with commercial PRRSV vaccines and inactivated farm-specific PRRSV vaccines was evaluated in PRRSV-immune, non-pregnant replacement sows from the 3 herds. A boost in virus-neutralizing antibodies against the farm-specific isolate was observed in all sow groups vaccinated with the corresponding farm-specific inactivated vaccines. Use of the commercial attenuated EU type vaccine boosted virus-neutralizing antibodies against the farm-specific isolate in sows derived from 2 farms, while use of the

commercial attenuated NA type vaccine did not boost farm-specific virus-neutralizing antibodies in any of the sow groups. Interestingly, the commercial inactivated EU type vaccine boosted farm-specific virus-neutralizing antibodies in sows from 1 farm. In the second part of the study, a field trial was performed at one of the farms to evaluate the booster effect of a BEI-inactivated farm-specific vaccine and a commercial attenuated EU-type vaccine in immune sows at 60 days of gestation. The impact of this vaccination on maternal immunity and on the PRRSV infection pattern in piglets during their first weeks of life was evaluated. Upon vaccination with the farm-specific inactivated vaccine, a significant increase in farm-specific virus-neutralizing antibodies was detected in all sows. Virus-neutralizing antibodies were also transferred to the piglets via colostrum and were detectable in the serum of these animals until 5 weeks after parturition. In contrast, not all sows vaccinated with the commercial attenuated vaccine showed an increase in farm-specific virus-neutralizing antibodies and the piglets of this group generally had lower virus-neutralizing antibody titers. Interestingly, the number of viremic animals (i.e. animals that have infectious virus in their bloodstream) was significantly lower among piglets of both vaccinated groups than among piglets of mock-vaccinated sows and this at least until 9 weeks after parturition. The results of this study indicate that inactivated farm-specific PRRSV vaccines and commercial attenuated vaccines can be useful tools to boost PRRSV-specific (humoral) immunity in sows and reduce viremia in weaned piglets.

Chapter 4 describes the use of 2 PRRSV isolates (08V194 and 07V063) for PK15$^{Sn\text{-}CD163}$ grown or MARC-145 grown vaccine development and the efficacy of these BEI-inactivated vaccines was evaluated in homologous or heterologous challenge (challenge with 08V194). 08V194-specific neutralizing antibodies upon two vaccinations were induced with both BEI-inactivated, MARC-145 grown viruses, while vaccination with BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown viruses mainly primed a virus-neutralizing antibody response. In the groups 08V194 PK15$^{Sn\text{-}CD163}$ grown and 08V194 MARC-145 grown, the duration of viremia was significantly reduced with approximately 2 weeks upon homologous challenge. Similar results were obtained with 07V063 PK15$^{Sn\text{-}CD163}$ grown upon heterologous challenge. 07V063 MARC-145 grown did not significantly influence the viremia upon heterologous challenge.

In **Chapter 5**, the main findings of this thesis are recapitulated and discussed.

SAMENVATTING

Het porcien reproductief en respiratoir syndroom virus (PRRSV) is een RNA virus dat betrokken is bij reproductiestoornissen (zwak- en doodgeboren biggen, vroeggeboorte en abortus tijdens late dracht) bij zeugen en respiratoire problematiek bij biggen van alle leeftijden, wat resulteert in een grote economische verliezen in de varkensindustrie. Een grote genetische variabiliteit is aangetoond onder de verschillende PRRSV isolaten en dit heeft zijn weerslag op hun virulentie, pathogeniciteit, immunogeniciteit,… Deze diversiteit is een groot probleem betreffende PRRSV preventie en controle. Het gebruik van de huidige commerciële vaccins in diverse vaccinatiestrategieën is de meest voorkomende methode om de klinische en economische impact van PRRSV te onderdrukken. Momenteel is er nood aan nieuwe en veilige vaccins die bescherming kunnen bieden tegen de PRRSV stammen die ontsnappen aan de immuniteit geïnduceerd door de huidige beschikbare commerciële vaccins. De doelstelling van deze thesis was het evalueren van de werkzaamheid van een geïnactiveerd bedrijfsspecifiek PRRSV vaccin - op basis van een eerder geoptimaliseerde virus- inactivatiemethode – en deze te vergelijken met die van commercieel beschikbare geattenueerde/geïnactiveerde PRRSV vaccins in naïeve en PRRSV-immune dieren. Tot op heden is het niet gekend welke voordelen er verbonden zijn aan het gebruik van deze bedrijfsspecifieke vaccins.

Hoofdstuk 1 geeft een korte samenvatting van de huidige literatuur over algemene aspecten van het virus en de ziekte, de PRRSV-specifieke immuniteit en PRRSV vaccins.

In **hoofdstuk 2** wordt de algemene doelstelling van deze thesis beschreven en worden een aantal doelstellingen vooropgesteld. De volgende 2 hoofdstukken omvatten onderzoeksresultaten die aan deze doelstellingen tegemoetkomen.

De eerste studie in deze thesis (**hoofdstuk 3 deel 1**) richtte zich voornamelijk op het vergelijken van de werkzaamheid van de homologe BEI-geïnactiveerde vaccins met heterologe BEI-geïnactiveerde vaccins en commerciële geïnactiveerde en geattenueerde vaccins na een homologe of heterologe challenge. De homologe BEI-geïnactiveerde vaccins zorgden na infectie voor een significante reductie in viremie. Ondanks de twijfel betreffende de werkzaamheid van de commercieel geattenueerde vaccins - die ook worden gebruikt op de bedrijven waar de PRRSV-isolaten vandaan

komen – hadden de commerciële geattenueerde vaccins toch een invloed op de duur van de viremie na infectie. De heterologe BEI-geïnactiveerde en commercieel geïnactiveerde vaccins daarentegen hadden geen of beperkte invloed op de viremie.

In een tweede luik (**hoofdstuk 3 deel 2**) werden de BEI-geïnactiveerde bedrijfsspecifieke vaccins vergeleken met commercieel beschikbare geattenueerde / geïnactiveerde vaccins in PRRSV-immune zeugen qua boosten van de humorale immuniteit tegen huidig circulerende PRRSV isolaten. In een eerste deel van deze studie werd de PRRSV-specifieke antistoffenrespons na booster vaccinatie met commerciële vaccins en BEI-geïnactiveerde bedrijfsspecifieke vaccins geëvalueerd in niet drachtige reforme zeugen van 3 bedrijven met PRRS-problemen ondanks vaccinatie van zeugen en gelten. Een boost in virus-neutraliserende antistoffen tegen de bedrijfsspecifieke PRRSV isolaten werd opgemerkt bij alle zeugen die werden gevaccineerd met het respectievelijke geïnactiveerde bedrijfsspecifieke vaccin. De commerciële geattenueerde en geïnactiveerde vaccins gaven gemengde resultaten qua boosten van virus-neutraliserende antistoffen tegen het bedrijfsspecifieke isolaat. In het tweede deel van de studie werd een veldproef uitgevoerd op 1 van bovenvermelde bedrijven om het booster effect van het BEI-geïnactiveerd bedrijfsspecifiek vaccin en het commercieel geattenueerd EU-type vaccin bij PRRSV-immune zeugen op 60 dagen dracht te evalueren. De impact van deze vaccinatie op de maternale immuniteit en op het PRRSV infectiepatroon bij de biggen gedurende hun eerste levensweken werd geëvalueerd. Na vaccinatie met het bedrijfsspecifieke vaccin werd een sterke stijging in bedrijfsspecifieke virus-neutraliserende antistoffen opgemerkt bij alle zeugen. Tot 5 weken na de geboorte werden virus-neutraliserende antistoffen gevonden in het bloed van biggen afkomstig van deze zeugen. Niet alle zeugen gevaccineerd met het commercieel geattenueerd EU-type vaccin toonden een stijging in bedrijfsspecifieke virus-neutraliserende antistoffen en de biggen afkomstig van deze zeugen hadden in het algemeen minder virus-neutraliserende antistoffen. Het aantal viremische biggen was significant lager bij biggen van beide gevaccineerde zeugengroepen dan bij biggen afkomstig van de controle zeugen en dit bleef zo tot op 9 weken na hun geboorte.

In **Hoofdstuk 4** werd de werkzaamheid van het BEI-geïnactiveerd vaccin virus geproduceerd op de nieuwe gevoelige cellijn PK15$^{Sn\text{-}CD163}$ vergeleken met het BEI-geïnactiveerd vaccin virus geproduceerd op MARC-145 cellen in een homologe en

heterologe situatie bij niet-immune biggen. In de homologe PK15$^{Sn\text{-}CD163}$ gegroeide en MARC-145 gegroeide BEI-geïnactiveerde vaccins, was na infectie de duur van de viremie significant gereduceerd met gemiddeld een tweetal weken. Een gelijkaardige resultaat werd bekomen met het heteroloog PK15$^{Sn\text{-}CD163}$ gegroeide vaccin. Geen significante reductie in viremie werd geobserveerd met het heteroloog MARC-145 gegroeid vaccin na heterologe infectie.

In **hoofdstuk 5**, worden de belangrijkste bevindingen samengevat en bediscussieerd.

CURRICULUM VITAE

PERSONALIA

Marc Geldhof werd geboren te Roeselare op 3 februari 1981. In 1999 beëindigde hij zijn studies secundair onderwijs in de richting Latijn-Wetenschappen aan het Heilig Hart College te Waregem. In datzelfde jaar startte hij zijn universitaire studies aan de Faculteit Diergeneeskunde en behaalde in 2005 de academische graad van dierenarts met voldoening.

Na het beëindigen van zijn studies, was hij gedurende ruim twee jaar actief als praktijkdierenarts in de dierenartsenpraktijken Degudap in Izegem en Eybecque in Boeschepe.

Vervolgens trad hij halfweg 2007 in dienst bij de vakgroep Virologie, Parasitologie en Immunologie bij het laboratorium voor Virologie. Hij verrichte er een onderzoeksstudie getiteld “Adaptable inactivated vaccine for combating Porcine Reproductive and Respiratory Syndrome Virus” onder leiding van Prof. Dr. H. Nauwynck. Dit doctoraatsonderzoek werd door de Federale Overheid gefinancierd. Naast het verrichten van onderzoek, was Marc ook medeverantwoordelijk voor de diagnose van virale ziekten bij het varken.

Eind 2011 was hij werkzaam als dierenarts varkensgezondheidszorg bij DGZ Vlaanderen. Vanaf 2012 werkt hij als varkensdierenarts bij voeders Ostyn in Roeselare. Zijn dagelijkse bezigheid is het begeleiden van varkensbedrijven met als doel een betere dierengezondheid en prestatiegerichtheid.

Marc Geldhof is auteur of mede-auteur van 13 wetenschappelijke publicaties in internationale tijdschriften. Hij nam actief deel aan nationale en internationale congressen.

PUBLICATIES EN WETENSCHAPPELIJKE MANIFESTATIES

PUBLICATIES IN INTERNATIONALE WETENSCHAPPELIKE TIJDSCHRIFTEN (A1)

Geldhof, M.F., Van Breedam, W., De Jong, E., Lopez Rodriguez, A., Karniychuk, U., Vanhee, M., Van Doorsselaere, J., Maes, D. & Nauwynck, H.J. (2013). Antibody response and maternal immunity upon boosting PRRSV-immune sows with experimental farm-specific and commercial PRRSV vaccines. *Veterinary Microbiology*, doi: 10.1016/j.vetmic.2013.08.017.

Geldhof, M.F., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Karniychuk, U., Nauwynck, H.J. (2012). Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. *BMC Veterinary Research*, 8(1): 182.

Karniychuk, U.U., Saha, D., Vanhee, M., **Geldhof, M.F.**, Cornillie, P., Caij, A.B., De Regge, N., & Nauwynck, H.J. (2012). Impact of a novel inactivated PRRSV vaccine on virus replication and virus-induced pathology in fetal implantation sites and foetuses upon challenge. *Theriogenology,* 78(7):1527-1537.

Nauwynck, H.J., Van Gorp, H., Vanhee, M., Karniychuk, U., **Geldhof, M.**, Cao, A., Verbeeck, M. & Van Breedam, W. (2012). Micro-Dissecting the Pathogenesis and Immune Response of PRRSV Infection Paves the Way for More Efficient PRRSV Vaccines. *Transboundary and Emerging Diseases,* doi: 10.1111/j.1865-1682.2011.01292.x.

Van Doorsselaere, J., **Geldhof, M.**, Nauwynck, H.J. & Delputte, P.L. (2011). Characterization of a circulating PRRSV strain by means of random PCR cloning and full genome sequencing. *Virology Journal*, 10(8): 160.

Karniychuk, U.U., Saha, D., **Geldhof, M.**, Vanhee, M., Cornillie, P., Van den Broeck, W. & Nauwynck, H.J. (2011). Porcine reproductive and respiratory syndrome virus (PRRSV) causes apoptosis during its replication in fetal implantation sites. *Microbial Pathogenesis*, 51(3): 194-202.

Vanhee, M., Van Breedam, W., Costers, S., **Geldhof, M.F.**, Noppe, Y. & Nauwynck, H.J. (2011). Characterization of antigenic regions in porcine reproductive and respiratory syndrome virus envelope proteins by the use of peptide-specific serum antibodies. *Vaccine,* 29: 4794-4804.

Van Breedam, W., Costers, S., Vanhee, M., Gagnon, C.A., Rodriguez-Gomez, I.M., **Geldhof, M.F.**, Verbeeck, M., Van Doorsselaere, J., Karniychuk, U. & Nauwynck, H.J. (2011). PRRSV-specific mAbs: supporting diagnostics and providing new insights into the antigenic properties of the virus. *Veterinary Immunology and Immunopathology,* 141: 246-257.

Costers, S., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., **Geldhof, M.** & Nauwynck, H.J. (2010). GP4-specific neutralizing antibodies: a driving force in PRRSV evolution. *Virus Research,* 154(1-2): 104-13.

Vanhee, M., Costers, S., Van Breedam, W., **Geldhof, M.F.**, Van Doorsselaere, J. & Nauwynck, H.J. (2010). A Variable Region in GP4 of European-Type Porcine Reproductive and Respiratory Syndrome Virus Induces Neutralizing Antibodies Against Homologous But Not Heterologous Virus Strains. *Viral Immunology,* 23(4): 403-13.

Karniychuk, U.U., **Geldhof, M.**, Vanhee, M., Doorsselaere, J.V., Saveleva, T.A., & Nauwynck, H.J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. *BMC Veterinary Research,* 6(1): 30.

Saha, D., Lefebvre, D.J., Van Doorsselaere, J., Atanasova, K., Barbé, F., **Geldhof, M.F.**, Karniychuk, U.U. & Nauwynck, H.J. (2010). Pathologic and virologic findings in mid-gestational porcine foetuses after experimental inoculation with PCV2a or PCV2b. *Veterinary Microbiology,* 145(1-2): 62-68.

Vanhee, M., Delputte, P.L., Delrue, I., **Geldhof, M.F.** & Nauwynck, H.J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Veterinary Research,* 40(6): 63.

PUBLICATIES IN VOORBEREIDING

Geldhof, M.F., Vanhee, M., Van Doorsselaere, J., Van Breedam, W. & Nauwynck, H.J. (2013). Porcine reproductive and respiratory syndrome virus variability on vaccinating pig farms in Flanders. *Manuscript in preparation.*

Geldhof, M.F., Trus, I., Delrue, I., Van Doorsselaere, J. & Nauwynck, H.J. (2013). Comparison of the efficacy of MARC-145-grown inactivated PRRSV vaccine virus and $PK15^{Sn\text{-}CD163}$-grown inactivated PRRSV vaccine virus against homologous and heterologous PRRSV challenge. *Manuscript in preparation.*

PRESENTATIES OP NATIONALE EN INTERNATIONALE CONGRESSEN

Vanhee, M., Delrue, I., **Geldhof, M.F.**, Delputte, P.L. & Nauwynck, H.J. New strategy to develop effective inactivated PRRSV vaccines. *2008 International PRRS symposium.* December 2008, Chicago (Il), USA.

Geldhof, M.F., Vanhee, M. & Nauwynck, H.J. Moeilijkheden bij bestrijding van PRRSV…op zoek naar nieuwe vaccinatiestrategieën. *IPVS Belgian Branch.* Oktober 2009, Merelbeke, Belgium.

Saha, D., Lefebvre, D.J., Van Doorsselaere, J., Atanasova, K., Barbé, F., **Geldhof, M.**, Karniychuk U.U. & Nauwynck, H.J. Clinical and virological outcome of experimental PCV2a and PCV2b infections in mid-gestational porcine foetuses. *21st International Pig Veterinary Society congress.* July 2010, Vancouver, Canada.

Geldhof, M.F. Virale ziekten: beknopte samenvatting van presentaties IPVS-congres Vancouver. *IPVS Belgian Branch.* September 2010, Merelbeke, Belgium.

Vanhee, M., Costers, S., Van Breedam, W., Van Doorsselaere, J., **Geldhof, M.,** & Nauwynck, H.J. GP4-specific neutralizing antibodies: A driving force in the evolution of porcine reproductive and respiratory syndrome virus. *10th International Conference on Moleculair Epidemiology and Evolutionary Genetics of Infectious Diseases.* November 2010, Amsterdam, The Netherlands.

Vanhee, M., Van Breedam, W., Costers, S., **Geldhof, M.F.**, Noppe, Y. & Nauwynck, H.J. Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use of peptide-specific serum antibodies. *3rd Meeting of the PoRRSCon Consortium.* January 2011, Madrid, Spain.

Geldhof, M.F., Karniychuk, U., Van Doorsselaere, J., Maes, D. & Nauwynck, H.J. Inactivated vaccines, experimental and at farm level. *3rd Meeting of the PoRRSCon Consortium.* January 2011, Madrid, Spain.

Karniychuk, U., **Geldhof, M.**, Vanhee, M., Saha, D. & Nauwynck, H.J. PRRSV challenge of late term pregnant gilts vaccinated with an experimental inactivated vaccine. *6th International Symposium on Emerging and Re-emerging Pig Diseases.* June 2011, Barcelona, Spain.

Saha, D., Lefebvre, D.J., Karniychuk, U., **Geldhof M.**, Ducatelle, R., Van Doorsselaere J. & Nauwynck, H.J. Outcome of porcine circovirus type 1 infections in mid-gestational porcine foetuses. *6th International Symposium on Emerging and Re-emerging Pig Diseases*. June 2011, Barcelona, Spain.

Saha, D., Karniychuk, U.U., **Geldhof, M.**, Ducatelle, R., Van Doorsselaere J. & Nauwynck, H.J. Exceptional outcome of an infection with a PCV2b strain in mid-gestational porcine foetuses. *XV International Congress of Virology, International Union of Microbiological Societies (IUMS).* September 2011, Sapporo, Japan.

Geldhof, M.F. Virale ziekten: beknopte samenvatting van presentaties/abstracts 6th International Symposium on Emerging and Re-emerging Pig Diseases. *IPVS Belgian Branch.* September 2011, Merelbeke, Belgium.

ABSTRACTS OP NATIONALE EN INTERNATIONALE CONGRESSEN

Vanhee, M., Delrue, I., **Geldhof, M.F.**, Delputte, P.L. & Nauwynck, H.J. Evaluation of a new experimental vaccine for the porcine reproductive and respiratory syndrome virus. *Belgian Society for Microbiology: Stress responses in the microbial world.* December 2008, Brussels, Belgium.

Karniychuk, U.U., **Geldhof, M.**, Vanhee, M., Van Doorsselaere, J. & Nauwynck, H.J. The current antigenic heterogeneity between PRRSV strains can influence the reliability of serological surveys. *2009 International PRRS Symposium,* December 2009, Chicago (Il), USA.

Karniychuk, U.U., **Geldhof, M.**, Vanhee, M., Van Doorsselaere, J. & Nauwynck, H.J. Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus strain. *2009 International PRRS Symposium,* December 2009, Chicago (Il), USA.

Geldhof, M.F., Vanhee, M., Van Doorsselaere, J., Karniychuk, U., & Nauwynck, H.J. Efficacy of an experimental inactivated PRRSV vaccine based on a recently isolated field strain. *2009 International PRRS Symposium,* December 2009, Chicago (Il), USA.
Also presented at: *21st International Pig Veterinary Society congress.* July 2010, Vancouver, Canada.
Also presented at: *IPVS Belgian Branch Symposium "Vaccination in pigs: Educate before you vaccinate",* November 2011, Ghent, Belgium.

Karniychuk, U.U., Saha, D., **Geldhof, M.**, Vanhee, M., Cornillie, P. & Nauwynck, H.J. Porcine reproductive and respiratory syndrome virus (PRRSV) replication in fetal implantation sites. *International Federation of Placenta Associations Meeting 2010*, October 2010, Santiago, Chili.

Geldhof, M.F., Van Doorsselaere, J., Vanhee, M., Van Breedam, W., Karniychuk, U.U. & Nauwynck, H.J. Genetic diversity of Porcine Reproductive and Respiratory Syndrome virus isolates in Flanders. *Conference of the Flemish Society for Veterinary Epidemiology and Economics "Eradication of endemic animal diseases: how far do we go?",* October 2010, Roeselare, Belgium.
Also presented at: *Belgian Society for Microbiology: Molecular dialogue in host-parasite interaction*, November 2010, Brussels, Belgium.
Also presented at: *IPVS Belgian Branch Symposium "Vaccination in pigs: Educate before you vaccinate",* November 2010, Ghent, Belgium.

Van Doorsselaere, J., **Geldhof, M.**, Nauwynck, H.J., & Delputte, P.L. Application of a random PCR cloning approach for the characterization of a circulating Flemish PRRSV strain. *Conference of the Flemish Society for Veterinary Epidemiology and Economics "Eradication of endemic animal diseases: how far do we go?",* October 2010, Roeselare, Belgium.

Vanhee, M., Costers, S., Van Breedam, W., Van Doorsselaere, J., **Geldhof M.F.** & Nauwynck, H.J. GP4-specific neutralizing antibodies: a driving force in the evolution of porcine reproductive and respiratory syndrome virus. *International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases,* November 2010, Amsterdam, The Netherlands.

Saha, D., Lefebvre, D.J., Van Doorsselaere, J., Atanasova, K., Barbé, F., **Geldhof, M.F.**, Karniychuk U.U. & Nauwynck, H.J. Pathologic and virologic findings in mid-gestational porcine foetuses after experimental inoculation with PCV2a or PCV2b. *IPVS Belgian Branch Symposium "Vaccination in pigs: Educate before you vaccinate",* November 2010, Ghent, Belgium.

Karniychuk, U.U., Saha, D., **Geldhof, M.**, Vanhee, M., Cornillie, P. & Nauwynck, H.J. Porcine reproductive and respiratory syndrome virus (PRRSV) causes apoptosis during its replication in fetal implantation sites. *IPVS Belgian Branch Symposium "Vaccination in pigs: Educate before you vaccinate",* November 2010, Ghent, Belgium.
Also presented at: *6th International Symposium on Emerging and Re-emerging Pig Diseases.* June 2011, Barcelona, Spain.

Vanhee, M., Van Breedam, W., Costers, S., **Geldhof, M.F.**, Noppe, Y. & Nauwynck, H.J. Identification of non-neutralizing and neutralizing antigenic regions in PRRSV by the use of peptide-specific serum antibodies. *The XIIth International Nidovirus Symposium.* June 2011, Michigan (Il), USA.

Karniychuk, U.U., **Geldhof, M.**, Vanhee, M., Saha, D. & Nauwynck, H.J. PRRSV challenge of late term pregnant gilts vaccinated with an experimental inactivated vaccine. *6th International Symposium on Emerging and Re-emerging Pig Diseases.* June 2011, Barcelona, Spain.

Geldhof, M., Karniychuk, U.U., Vanhee, M., Van Doorsselaere, J., Vandersmissen T., Maes, D. & Nauwynck, H.J. Use of a farm-specific inactivated vaccine for boosting the humoral immunity of infection-immune sows in comparison with the current available commercial vaccines. *6th International Symposium on Emerging and Re-emerging Pig Diseases.* June 2011, Barcelona, Spain.

Geldhof, M., De Jong, E., Lopez Rodriguez, Van Doorsselaere, J., Maes, D. & Nauwynck, H.J. Efficacy of a farm-specific inactivated PRRSV vaccine in the field. *6th International Symposium on Emerging and Re-emerging Pig Diseases.* June 2011, Barcelona, Spain.

Geldhof, M., Delrue, I., Van Doorsselaere, J. & Nauwynck, H.J. Comparison of the efficacy of inactivated PRRSV vaccine virus grown on MARC-145 or PK15$^{SN-CD163}$ cells. *6th International Symposium on Emerging and Re-emerging Pig Diseases.* June 2011, Barcelona, Spain.

Garcia Duran, M., Sarraseca, J., Roja, N., Vanhee, M., **Geldhof, M.**, Vela, C. & Rodriguez, M.J. PRRSV nsp7 as a candidate target protein for serological differentiation between infected and vaccinated animals (DIVA assay). *6th International Symposium on Emerging and Re-emerging Pig Diseases.* June 2011, Barcelona, Spain.

Vandersmissen, T., Brossé, C., de Jong, E. & **Geldhof, M.** Insights in *Brachyspira hyodysenteriae* on nine Belgian pig herds. *5th European Symposium of Porcine Health Management*. May 2013, Edinburgh, UK.

PATENTEN

Delputte, P.L., Nauwynck, H.J. & **Geldhof M.F.** Cross-protecting vaccine for porcine reproductive and respiratory syndrome virus. EP/16.04.10/ EPA 10160139.

DANKWOORD

)

**At the end of the day faith is a funny thing. It turns up when you don't really expect it.
It's like one day you realize that the fairy tale may be slightly different than you
dreamed. The castle, well, it may not be a castle.
And it's not so important happy ever after, just that it's happy right now.
See, once in a while, once in a blue moon, people will surprise you,
and once in a while people may even take your breath away.**

M. Grey

Eindelijk is het dan zover… Het enige wat me nu nog rest is het dankwoord…

Toen ik afstudeerde, had ik nooit kunnen vermoeden dat ik ooit nog aan de faculteit zou werken, laat staan dat ik zou doctoreren. Zo zie je maar, het leven zit vol onverwachte wendingen (mits een beetje hulp van Annick ☺).

Een doctoraat maken doe je niet alleen. Ik beschouw dit werk dan ook als het resultaat van "grosso modo" viertal fijne jaren teamwerk. Gedurende deze periode is het soms moeilijk de motivatie te vinden om verder te gaan en dan is het een steun te weten dat je een vangnet hebt van mensen die om je geven. Nu ik de faculteit ondertussen 2 jaar heb verlaten (besef ik hoe speciaal en bijzonder m'n tijd hier is geweest), zou ik de mensen die deze taak voor hun rekening hebben genomen graag willen bedanken.

Eerst en vooral wil ik mijn promotor Prof. Dr. Hans Nauwynck bedanken.
Dank je wel voor uw enthousiaste ideeën en geanimeerde gesprekken. Ik apprecieer enorm de tijd die je in de laatste maanden voor mij hebt vrijgemaakt, en ik weet dat dit niet evident was met een kleine 20 (misschien zelfs meer) doctoraatsstudenten onder jouw hoede. Bedankt alsook voor het vertrouwen in een goede afloop.

Ook dank ik de Federale Overheidsheidsdienst Volksgezondheid voor het financieren van dit project, waardoor het mij mogelijk werd gemaakt te doctoreren.

Ik wil de mensen uit mijn begeleidingscomité danken voor het kritisch nalezen van dit doctoraat. Prof. E. Cox, Prof. D. Maes en Dr. W. Van Breedam, bedankt voor jullie kritische kijk en handige tips. I also would like to thank the other members of the exam committee: Prof. Dr. M. Ritzmann, Prof. Dr. K. Van reeth, Dr. B. Caij, Dr. T. Meyns and J. Hooyberghs for the proofreading of this thesis and their constructive criticism. Jullie opmerkingen droegen zeker bij tot de kwaliteit van deze thesis. Prof. K. Hermans, bedankt voor je inzet voor de ethische commissie.

Jan "tis hier Roeselare" (aan de telefoon) Van Doorselaere zou ik graag bedanken voor de bijzonder aangename samenwerking op meer moleculair gebied. Dank je wel Jan, voor het sequeneren van alle PRRSV isolaten, voor het vele over-en-weer gemail en de aangename bezoekjes in Roeselare. Succes met de start van een nieuw hoofdstuk aan het Katho.

Volgende mensen verdienen niet 1 bloemetje maar een gans bloementapijt, niet 1 pralineke, maar ganse dozen vol pralinekes:

Chantal, Carine, Lieve, Nele, Melanie, Dries, Tim, Ytse, Bart, Zeger, Gertje, Mieke, Ann, Marijke, Dirk, Magda en de oude (maar niet vergeten) garde Geert, Fernand en Chris: jullie zijn/waren de rots waarop de Virologie is gebouwd. Jullie inzet, werklust en enthousiasme voor de doctorandi is/was onovertroffen. "Bergen werk " (den Himalaya of het oeralgebergte of gelijk welken berg verdwijnt in het niets hierbij, geloof me) is er door jullie verzet in het kader van mijn project.

Chantal: bedankt voor het opgroeien van alle PRRSV stammen (wat niet altijd verliep zoals gepland met enkele "niet-groei-minded" opstandige, PRRSV isolaten), het uitvoeren van vele SN-testen en onnoemelijk veel virustitraties.
Carine: bedankt voor het uitvoeren van de talloze IPMA's en voor de hulp met de talrijke diagnosen. In het laatste geval waren we af en toe ook Sherlock Holmes en Dr. Watson op onderzoek naar wat voor opgestuurd stukje orgaan we voor onze neus hielden…☺. Beiden ook bedankt voor de fijne amusante babbels als we naast elkaar aan de flow zaten/of in de keuken ons boterhammekes aan het eten waren.
Nele en Melanie, bedankt voor het verwerken van de veelvuldige bloedstalen uit de veldproeven. Het was altijd uitkijken naar wanneer de stalen in het labo werden binnen gegooid. Melanie, ook bedankt voor het verwerken van alle paperassen gerelateerd aan het diagnosewerk. Nele, het was niet alleen heel aangenaam vertoeven met jou in het labo, maar ook buiten het labo: ik denk vooral aan alle filmavondjes (inclusief pitta in de overpoort), de gezellige avondjes uit, gaan badmintonnen of Zumba'en in de Ucon, enz…..Het allerbeste gewenst met uw schatje Eric in jullie nieuwe stekje.
Dries, bedankt voor de af en toe op het toneel verschijnende, noodzakelijke Western-blotjes uit te voeren. En ook voor alle leute en plezier (boerderijke, BBQ's, …) die we hebben beleefd buiten het labo.
Dan zitten we nog maar halfweg het uitgevoerde werk. Chantal, Nele, Melanie en Dries ook bedankt voor de "en mass productie" van virus voor de aanmaak van vaccinstocks. Hoeveel 50 mL flessen zouden we er niet doorgejaagd hebben?
Dries, Nele en Melanie bedankt voor het meehelpen met de opzuivering van het "en mass" geproduceerde virus. De Ultracentrifuge (is een machien) weet dankzij ons wat overuren "draaien" nu echt betekent.
Lieve, bedankt voor het uitvoeren van alle niet-standaard diagnosen. Kgaan u vooral missen, 's morgens met ons zjatte koffie in de keuken terwijl we commentaar gaven op de laatste pagina van de sport ☺.
Chris, jou wil ik bedanken voor het uitvoeren van de eerste IPMA's en de gezellige babbels. Je tijd op de virologie zat er al op, maar je kwam terug voor ons "niet lang in stand gehouden koortje" met het moment-supreme op de nieuwjaarsreceptie 2010". Ik weet zeker dat je je hierna niet zal vervelen met je kleinkind (eren), je huidige koor-activiteiten, reizen, ... Ik wens je het allerbeste.
Tim, jij bent de laatste aanwinst in het labo en vanaf dag 1 stond ge al direct uw mannetje. Ge draaide al direct mee in de "wetenschappelijk niet-verantwoorde" discussies in de keuken. En als ge ooit nog eens een partijtje sjotten organiseert, moogt ge dezen ancien altijd nog oproepen. En mercikes voor het gebruik van uwen computer in het moleculair labo!
Ytse, jij bent als stagiair gestart bij de PRRSV-groep (ocharme Merijn) en daarna overgeschakelt naar de FIP-groep (ocharmde de FIPpers). Ge zijt een straffe madam en uw zalig graptje van Bing met achternaam O op het kerstdiner van 2011 zal ik ni rap vergeten ☺.
Geert(je), bedankt voor alle hulp bij het uitvoeren van de (niet al te kleine) dierproeven: bloednames met ons tweekes => eer de varkens het beseften waren ze al 10 mL bloed kwijt ☺, we vlogen erdoor (zoals raketman in de Humo geweest), maar in het kuisen van de stallen, bleken we dan weer geen crack in te zijn ☺. Ge waart nen aangename kerel: in de stallen tussen de varkens zijn er vele topics de revue gepasseerd en je had altijd wel een leuke quote om in het gesprek te gooien. Ik hoop dat onze regelmatig geplande etentjes nog lang door blijven gaan.

Fernand, jij was destijds de Influenza-kerel en een nauwe samenwerking was dus niet voor ons weggelegd! Ik wens u een welverdiende rust en geniet van uw pensioen!
Bart en Zeger, jullie zijn de opvolgers van het schitterende duo Geert en Fernand! De start in het labo was misschien niet wat het moest zijn, maar dit is uiteindelijk toch goedgekomen! Jullie waren goede "studenten" bij het aanleren van bloednames bij zowel biggen als zeugen (het was toen efkens geleden, dat ik nog zo'n jeugdig enthousiasme zag). Jullie hulp was van grote waarde bij mijn laatste dierproeven. We kunnen al enkele straffe verhalen vertellen over de zeugen a/d overkant (de "pony" en die ene keer met Hossein mee ☺)! Zeger en Aludee, het allerbeste gewenst!
Magda, ook jou zou ik willen bedanken voor uw goedbedoelde bezorgdheid voor alle beestjes, het kuisen van de stallen en voor het regelen van het transport naar de patho voor de platgespoten varkentjes.
Gert(jeuh), in alle dankwoorden wordt je bedankt voor de bestelde productjes en antistoffen; ik heb dus bijna nikske besteld dus kan ik dit "standaardzinnetje" niet gebruiken. Ik kon wel altijd rekenen op jouw zeer bereidwillige medewerking om de facturen van de biggen in orde te brengen en om me te tonen waar ik extra fluostiften kon vinden ☺. Maar naast je professionele prestaties werden uiteraard ook de babbels, de mopjes, de opmerkingen in de keuken, de educatieve muurversieringen, de Kinepolis@gertjeuh-avonden… zeer zwaar geapprecieerd (doe zo verder!). En nog heel veel mooie papa-momenten met Lucas gewenst!
Mieke, jij draagt zorg voor de administratieve (in de breedst mogelijke betekenis van het woord, den Dikke vandaele kent nog niet al die betekenissen) zaken. Het in orde brengen van trein- of vliegtuigticketten, enz….was een kolfje naar je hand. Dank je wel, Mieke, voor de aangename samenwerking.
Ann, bedankt om de financiële kantjes van het doctoraat (bij iedereen dus) in orde te brengen.
Ook Marijke zou ik graag bedanken voor het proper houden van onze werkvloer en onzen bureau, maar vooral ook voor de leuke babbels over vanalles en nog wat, maar vooral die ene terugkerende topic: Cavia's. Merci, om destijds 2 van die koterkes (geboren na een onbelvekte ontvangenis van 1 van mijn cavia's) een schitterende thuis te geven en aan uw verhalen te horen zijn ze in het aardse paradijs beland!
Dirk, jou zou ik willen bedanken voor de steun op computergebied gedurende de 4 jaren van het doctoraat en voor het bestellen van de oornummers (bestemd voor de varkentjes in de veldproeven). Het laatste jaar stond ik toch heel frequent aan uwen bureau met "een voor mij onmogelijke, maar voor u poepsimpele" computervraag. Ik dacht dat jouw vinnige opmerking en commentaren gingen verdwijnen, nadat ik me had aangesloten bij het "I♥"-team, maar dat bleek ijdele hoop ('t werd nog erger ☺).

En dan komen de ex-bureaugenootjes aan de beurt…
David en Debby, de eersten die een bureau (of wat toch zou moeten doorgaan als een bureau) met me deelden. David de "ancien" , Debby en ik de groentjes… Bedankt voor de soms ontspannende, soms serieuze, soms wetenschappelijke babbels en de steun tijdens de eerste 2 jaren. Allebei veel succes in jullie verdere carrière en privéleven (aan de regelmatige updates te horen is dit geen probleem ☺). En Debby, hopelijk nog heel veel eitjes van de "gewisselde kip" ☺.

Na jullie vertrek werden de grote hervormingen (waarvan lang sprake geweest) in het labo uitgevoerd en werd ik verscheept naar een nieuwe locatie met nieuwe bureaugenoten en die nieuwe garde was niet van de poes zenne: Sarah C., Merijn en Wander. Wat betreft PRRSV in al zijn moleculaire/immunologische facetten zijn julie

in mijn ogen de crème de la crème. Naast de wetenschappelijke discussies werd er ook (gelukkig) veel onnozele praat en gezever verkocht (voorlopig behoort dit tot de categorie unpublished data) in den PRRSV-bureau. Ik denk dat we toch een aantal boeken met fantastische verhalen zouden kunnen schrijven (misschien iets voor na onze "wetenschappelijke" carrieres…..).
Merijn, de allrounder van onzen bureau. Toen ik arriveerde op het labo ben ik onder uw vleugels terchtgekomen (niet letterlijk) en heb je me alles geleerd van het arrangeren van dierproeven tot het opzuiveren van virus. Ge waart de best mogelijke opvang die iemand zich kon wensen (zoals het OCMW in België is voor de vreemdelingen). Nen dikke merci voor alles en wat ik nu vooral mis zijn uw te pas en te onpas flauwe/droge mopjes ☺. En ons neushoorn/bibliothecaris moment zal ik ook niet rap vergeten ☺. Heel veel succes op het Katho, met de nieuwe woonst (hopelijk snel een nieuwe buurman), Sofie, Jens en Nina.
Wander, de perfectionist en het "stress-konijn" van de bureau (nu al een stuk minder na een pracht van een doctoraat), ik bewonder uw alwetendheid, ge zijt echt nen wandelende Wikipedia. En mercikes om ook mijn co-promotor te willen zijn! Zonder U en Merijn zouden de artikels en PhD nog ergens stof liggen vergaren in een vergeten hoekje! Nog eens nen dikke merci X 10^6! Ik wens u vanaf nu een fantastische post-doc periode, waarin je een volledige vrijheid hebt qua uitvoering ervan. Geniet van de toekomst met Leslie in jullie knusse appartementje.
Sarah C., jij werd direct gebombardeerd tot de alwetende moederkloek (en niet enkel door mij alleen, had ik den indruk). Met iedere vraag konden we bij u terecht. Je had den bureau en (gebroken) stoel van Peter dubbel en dik verdiend! Op het laatst kende ge zelfs het wereldje van de Gentse vastgoedmarkt gelijk uw broekzak (hoe je de verhuurders aanpakte ad telefoon, jadadde). Bedankt voor het introduceren van Loezendag in den bureau! En het record van "het hardst mogelijk toeslaan/toesmijten van een proffendeur" staat nog altijd op uw naam ☺. Veel succes in de toekomst met uw prachtig gezinneke, al uw poezen en de "nieuwe" job.
Na de rouwperiode van Sarah's vertrek werd Angela bij ons in de bureau binnengegooid. Angela, i don't know where to start! I'm really happy you shared the office with us. When Merijn and Wander had very scientific discussions on a higher level and we were involved, we could exchange a glance saying "i really don't understand anything they say" and then we just nodded yes…☺ And then the moments we were alone in the office and the telephone rang…. ☺☺. I wish you all the best and a shining fantastic future with Michael.

De meeste collega's die de Virologie bevolkten gedurende mijn PhD-tijd zijn reeds vertrokken. Hierbij volgt nu en lijstje van bijna allemaal Doctors die een fantastische blijvende indruk op mij hebben gemaakt en die ik zeker niet zal vergeten:
Annick, zoals eerder aangehaald ben ik via u het labo binnengerold… (Ze hadden eigenlijk nen Roemeen nodig, maar blijkbaar voldeed ik toch min of meer aan de voorwaarden ☺). Ons verleden gaat terug tot aan 1ste kan Diergeneeskunde (kzie ons daar nog altijd zitten in de auditoria van de Ledeganck). Sindsdien zijn we heel goede vrienden gebleven met heel leuke anekdotes tot gevolg en die je altijd heel sappig kunt vertellen ☺. Bedankt om al die jaren al een goede steun te zijn! Veel succes met uw praktijk, uw DGZ-avontuur en ook met Jan-Bart, Aline en de nieuwe kleine spruit.
Annelies, bedankt voor de leuke babbels, de slappe-lach momenten en ons uitstapje naar de jobbeurs in Brugge ☺. Daar hebben we toch wel geduld leren kweken…☺. Ik wens u het allerbeste bij DGZ (tot ne volgende babbel aan de poort) en met uw ventjes Lieven en Bas. Ook succes met de verbouwingen in jullie huisje.

Filip, gij waart de oplossing voor mijn interferon-vraagjes! Hieruit zijn de aangename babbels (zowel wetenschappelijk als niet-wetenschappelijk) voortgevloeid! Ik heb echt genoten van uw gezelschap en ge kon mij toch soms met verstomming slagen door in een gesprek straffe uitspraken te doen; (van zo'n fatsoenlijk iemand had ik dat immers ni verwacht ☺) Ook jou wens ik veel succes toe met uw "nieuwe" job, met Elise en met uw dochterje Ilona.
Sarah G (of Cherry, zoals Sabrina je noemt), jij bent echt een fantastische meid! Je staat altijd klaar voor anderen en je hebt een eindeloos geduld! Ik heb ontelbare keren jouw zelfbeheersing bewonderd! En nog meer bewondering als ik hoorde dat je toen bleef voor een post-doc positie, jadadde…. Bedankt voor de leuke, meestal avondlijke, babbels over vanalles-en-nog-wat. Ik wens u een fantastische toekomst met uw ventje Joost en een fantastische zwangerschap.
Sabrina (Miss Italy), despite all the misery in different areas the last years, you approached everything from an optimistic point of view and giving up is not written in your dictionary! You are the most energetic woman, I've ever met in my whole life. I'm really happy that most things are cleared out now and i will always remember you with a big smile. I wish you a great time with your daughter Alicia!
Sjouke (of Sjoe), één van de straffe madammen in het labo! Mercikes voor de vele deugddoende babbels en jouw bereidheid om te helpen waar je kon (lees: helpen bloedtrekken in het weekend, assisteren bij de diagnoses (wat door 'sommigen' niet in dank werd afgenomen ☺), de toffe tuinfeestjes, de hulp bij slachtpartijen onder de varkentjes en nog zoveel meer... Ik wens u een schitterende toekomst met Bart en een superstart op de nieuwe job!
Zhongfang, you are a very special and exceptional Chinese guy! You really have a funny humor (remember our phonecall…☺). You surprised me (and my mouth/throat/stomach) with your (spicy) cooking skills. I also wish you the best with your Chinese vases collection and all the things you plan to do in the future.
En wie zou onze Brielse madam Veerle kunnen vergeten. Bedankt voor alle leute en nen hele dikke chapeau voor de beslissing qua carrierewending. Ik wens u het allerbeste in de toekomst en hopelijk ben je al wa gewend aan hetvolgende: Mevrouw Billiau, ik heb mijn huiswerk niet bij want de hond heeft het opgegeten… Ik hoop van ganser harte dat we met "ons bendeke" nog veel "komen eten avondjes" mogen meemaken!
Miet, nog een straffe madam uit ons labo! Uw onderzoek is zeker niet over rozen gelopen en toch ben je blijven volhouden! Da getuigt van een sterk karakter! Daarnaast wil ik u bedanken voor de vele babbels, uw luisterend oor, de etentjes bij u thuis (da gedoe met de lift en de 6 brandweermannen zal ik nooit ni vergeten ☺), de last-minute etentjes om ons hartje eens te luchten, de leuke avondjes uit en zoveel meer… Ik hoop dak nog veel van u mag horen in de toekomst!
Nina, je bent dezelfde dag als mij begonnen op de faculteit en ik zie ons daar nog zitten op onze eerste dag in de vergaderzaal ☺. Ondertussen ben je al Dr. Post-doc Nina met bijna nen Nature. Nen dikke Chapeau! Bedankt voor de leuke babbels aan de flow over fantasy-boeken (de wetten van de magie; Harry Potter), de toffe squashmomenten (I kicked your ass, baby) en de vele babbels tussendoor. Een fantastische toekomst gewenst met Guillaume en Pixie.
Hanne (onze PRRSV-First-Lady), Dank je wel voor de aangename babbels, jouw moleculaire expertise en de leuke sfeer tijdens de Journal clubs! Hopelijk veel bijgeleerd in den Ameriek en hopelijk kwam de gsm ginder goe van pas! Veel succes met de "nieuwe" job op het VIB en jullie nieuw stekje ☺.

Evelien, ook jij was zeer belangrijk voor de opperbeste sfeer in het labo! Ik zal nooit niet het moment vergeten dat ge plots een stemmetje hoorde toen je op het toilet zat…☺. Ook bedankt voor een goe jaar mijn spinning-partner te zijn op maandagavond. Het allerbeste met Lieven, uw oogappel Juliette en uw job!
Leslie (de muze van vele onderzoekers en 1 boekhouder), jij bent overgeschakeld naar de pathologie. Na uw vertrek voelden we ons precies Bambi in die scene waar hij zijn mama niet meer vindt…☹. Je bent opengebloeid nu je schattige (dode) beestjes in stukken mag snijden/hakken/kappen. Doe dat nog goed in de pathozaal en alvast heel veel succes voor het residency-examen. Geniet van de toekomst met Wander in jullie knusse appartementje.
Mieke V., ik bewonder uw besluit om het doctoraat stop te zetten. Zo'n beslissing maken loopt niet van een leien dakje! Succes gewenst met de volgende carrierestap in uw leven. En wie weet komen we elkaar nog eens tegen op de Kouter voor nen voetbalmatch op groot scherm (en lok dan weer geen gevecht tussen hooligans uit, zoals de vorige keer ☺!).
Nick, bedankt voor de gezellige niet-wetenschappelijke babbels (al of niet over het konijn, of over het vissen, of over fitness), zowel in het labo als bij de talrijke buitenlabo'se activiteiten. Ik wens je veel succes toe met Ilse, jullie nieuw 'klein' konijntje en met uw job op het CODA.
Kalina, it was really great to have you in the lab, and to hear you talking, laughing, and singing in the lab late in the evening! You were a fantastic friend to go to the gym with! I can tell a whole bunch of stories of things that happened in the gym, which always ended in a big laughter ☺. Thanks also for a whole lot more (spending Christmas together, going to the movies, …). I wish you the best in everything you undertake on the other side of the big water!
Matthias D., jou wil ik bedanken voor de leuke West-vloamse babbels. En ook bedankt om me af en toe een oppepmail te sturen! Veel succes in het CODA!
Annebel, bedankt om een fijne collega te zijn op het labo en ook op de vele activiteiten daarbuiten! Uw oneliners zijn ondertussen ook al legendarisch ☺! Ook bedankt om me "efkens" onderdak te verschaffen, wanneer ik een plaatske zocht! Ons slappe-lach moment op feestje van Sabrina (money-girl) zal ik ook nog lang onthouden. Het allerbeste en een fantastische schone toekomst gewenst!
Dipo (Mister Bangladesh), I will never forget our trip to Vancouver, our trip to Barcelona, the (exciting) surgeries on the sows and all the fruit you can eat! Good luck with your Post-Doc in Finland.
Uladzimir, you're a guy who sticks to his own plan and i admire that! Keep organizing good barbecues with tasty 'out of the blue' meat ☺! Good luck in Canada and with your future research….
Ben, gij moest uw mannetje staan tussen al die vrouwen! Nen dikke chapeau (ik zou da ni kunnen zenne)! Ondertussen nog eens naar de Ghost and the Darkness gezien ☺! Succes met de nieuwe job en in alles dat je doet.
Hannah (the former leading lady of FIP), jou wil ik bedanken voor de leuke babbels en voor het stukje chocolade die ik ooit eens kreeg van de stapels chocolade-tabletten die op uw bureau lagen. Jij bent in mijn ogen het toonbeeld van een kalm en rustig persoon (in alle situaties). Succes met de "nieuwe" job en nog veel genot van/met uw gezinnetje.
Sabine (Miss Germany), you're a girl with a lot of knowledge (in my eyes)! Thanks for the nice talks and again a sorry for all my 'bad german jokes'. I will take care of better jokes in the future ☺. Good luck with finishing your residency, with Alejandro and you're little star Paulina (and the dogs of course).

Joao, i am very glad i had you as my colleague. Thanks to you i learned some new pubs and restaurants in Ghent! I will never forget your face when we were locked up in the elevator after having dinner @ Miet's place. I hope that in the near future we can have a 2nd dinner with goat on the menu! I wish you the very best with your spin-off and the fatherhood. You will do it fantastic!
Matthias C wil ik bedanken voor de leuke filosofische babbels en de avondjes uit in de overpoort! Ook een bedankje voor het adopteren van een overtollig dwergkonijn (en naar horen zeggen smaakte het erg lekker ☺). Ik wens je verder nog veel succes in alles wat je doet!

En nu de collega's die momenteel nog op de dienst virologie werken:
Lennert, waar begin ik mee… Ge zijt nen fantastische maat/sportbuddy/collega. Bedankt voor de vele lachbuien, de vele babbels, onnozele praat verkopen op het labo of in de Ucon/Coupure na/gedurende het sporten. Ik zal de happy hours en ons party-crash moment in den Aula (van de rechten) alleszins niet vergeten (diene blik van de rector… ☺)! De uitjes met Maria en Jasper zullen me ook altijd bijblijven en hopelijk volgen er nog… Ik wens u en Jasper een schitterende toekomst toe in den Antwerp (zo ver weg, jong)! Tot nog eens op het squashveld, als mijnen enkel het toelaat ☺!
Amy (Professor Mucus ☺), thx for all the unexpected funny remarks and for letting me taste all the typical Chinese food @ Chinese Newyear! I hope you're having a great time here with your husband Ming and good luck with finishing your PhD! You will do great ☺!
Hossein, you're one the friendliest people I have ever met! Good luck with searching the dendritic cells… And don't forget to bring your dancing moves, the next time we go out!
Ilias (Mister Greece), you're a great guy! I hope you have found your way in PRRSV research and good luck with your experiments! Thanks for the many talks and see you (maybe) at the gym!
Isaura, ge zijt een straffe madam met het hart op de tong! Succes met uw onderzoek!
Kathlyn, merci pour les gentil conversations et bonne chance avec votre recherche!

Als we naar beneden afzakken (en kbedoel in het labo) komen we uit in het FIP / targeting bureau:
Dominique, nog zo'n straffe madam! bedankt voor de vele leuke babbels, uw luisterend oor, de brunches en de sportmomenten in stadium Coupure. Succes met het finaliseren van uw onderzoek (ge zijt er bijna jippie!!!!! ☺). Tot ons volgende toerke lopen aan de watersportbaan om ons hartje eens te luchten ☺. Een super fantastische toekomst met Michael gewenst!
Karen, ge zijt ook één van de straffe madammen in het labo! Bedankt voor de fijne babbels, uw luisterend oor en voor het opzij houden van de koekjes (voor uw verjaardag), het voorschieten bij den drukker ☺ en al de support (de vele telefoontjes) de laatste weken! En ni vergeten: we moeten nog altijd eens een danske placeren hé ☺! Veel succes met de verbouwingen, met uw ventje en volgende week legt ge een megafantastisch doctoraat af. Kduim alvast ☺!
Annelike, bedankt om een toffe leuke collega te zijn en veel succes met het finaliseren van de PhD, de zwangerschap en alles wat je van plan bent!
Sebastiaan (de nieuwe Harry Potter), veel succes met het opzetten van uw darm-explant modellen (vergeet de cola-versie niet) en hopelijk hou je geen 'vliegende spetter' over aan uw onderzoek ☺. Succes met het koersen en uw onderzoek!
Lowiese (van Vichte), ge zijt een madam die niet op haar mondje is gevallen! Op het kerst-etentje zijn we te weten gekomen da ge graag bananen eet! Eet er toch niet

teveel en gunt uw vriend ook nog ietske hé ☺. Succes met uw verdere onderzoek!

Dan komen de mensen van Boven (degene die overal/nergens bij horen) nog aan de beurt:
Maria (Miss Portugal 2012), where do i have to start… Thx for all the animated discussions, for always telling the truth (sometimes in a rude way ☺), for taking me out on the worst dinner ever (place near the castle ☺☺), for having a lot of fun, for Starbucks on Saturdays, for sporting together, and many more things… I'll never forget our going out moments with Lennert and Jasper. Good luck with finishing your PhD. And also a little word for your bunny Rupa (the most spoiled rabbit I know): make the life of your bossy as difficult as possible ☺.
Jochen, ge staat min of meer aan de beginjaren van uw onderzoek. Blijven op de tanden bijten is de boodschap. Bedankt voor de vele babbels, de etentjes gepaard met een stapke-in-de-wereld-zetten, en nog zoveel meer. Da we de "traditie" van den Starbucks op zaterdag om wa bij te klappen nog lang mogen verderzetten ☺.
Céline, gij zijt altijd goedgezind en altijd bereid om hier en daar een handje te helpen als er ietske wordt georganiseerd. Bedankt hiervoor! En btw, ge zijt waarschijnlijk de snelste vrouw op ne vélo, die ik ken.
Cliff, gij moet den boel ginderboven een beetje rechthouden en ge doet da goe ☺! Thx om op mij te wachten om naar despicable me II te gaan zien! Kvond da chic! Tot de volgende meeting en het allerbeste met uw nieuw aanwinst (=leuk katje).
Thary, bedankt voor al het lekkers die je heel frequent meebracht naar het labo (misschien ben ik hierdoor wel in de oranje zone gesukkeld…☺). Een fantastisch huwelijk gewenst voor volgend jaar!
Herman, je staat altijd voor iedereen klaar om wetenschappelijke vragen te beantwoorden of om mee na te denken over mogelijke experimenten. Jouw hulpvaardigheid, ervaring en kennis zijn van grote waarde voor het labo. En btw, ik was sterk onder de indruk van uw conditie, daar ik getuige mocht zijn van een squash-wedstrijd (≈Federer versus Nadal waardig) tussen Lennert en u.

Een woordje van dank gaat ook uit naar iemand die een korte tussenstop heeft gemaakt op de Viro: Professor Dr. (en nog nen titel of 5☺) Gerlinde Vandewalle. Bedankt om altijd in mij te geloven en soms het positieve te laten zien waar ik het niet zag (onzen babbel op den trouw van Annelies en Lieven). Daarnaast bedankt voor de fijne babbels en voor uw fijn gezelschap op The Mission 2011.

In de loop van de jaren, zijn er ook buitenlandse "tijdelijke passanten" de revue gepasseerd op het labo:
Elisa, thx for all the fun/going out moments, for the football game (I liked when you said i was better than you expected ☺), for being our guide in Torino (Wedding Sabrina). I wish you a fantastic future and i hope to see you again.
Irene, we really laughed a lot with stupid things and thx for the really nice time @ the congress in Barcelona!
Eva B., thx for the nice talks and for being so colorfull.

Hierbij wil ik ook nog alle overige (vroegere) (ex-) collega's, zowel inheems (Karl, Inge (2x), Iris, An S., Ann D., Els, Korneel) als uitheems (Tù, Lang, Liping, Wenfeng, Yu, Charlie, Kevin, Constantinos, Gerald,…) bedanken voor de de gezellige sfeer op het labo! En al de mensen die ik vergeet: ook bedankt natuurlijk!

Nog eens allen bedankt voor alle toffe momenten (BBQ's, The Mission, de Quick, Don Pepe, komen-eten, Mardi Gras @ Belgaqueen, labo-uitstappen, een pintje de vrijdagavond, filmavondjes, weekendje ardennen, frietjesmoment, vuurwerk Knokke,…) op en meestal buiten het 2de verdiep van de hoogbouw!

Op de faculteit lopen/liepen er nog enkele mensen rond die ook een woordje van dank krijgen voor de hulp met het uitvoeren van de veldproeven: Alfonso, Ruben, Ellen, Ruben D., Ilse, Annelies, Emily, Josine, kortom de mensen van de overkant (Dienst Verloskunde). Allen een fantastische toekomst gewenst!
Ellen, het allerbeste bij DGZ en tot de volgende meeting!

In Memoriam: bedankt aan alle varkentjes (zowel biggetjes, vleesvarkens en zeugen) die (altijd ongewild) hebben meegewerkt aan dit onderzoek! Vooral Knorretje I en II verdienen postuum een extra bloemetje ☺.

Ook buiten de faculteit zijn er heel wat vriend(inn)en die ik graag zou bedanken voor hun interesse en steun gedurende de jaren van het doctoraat:
Gert, bedankt voor de vele telefoontjes/bezoekjes/etentjes… de afgelopen jaren. Het allerbeste met uw vrouwke Sarah, en jullie klein kadetten Joppe en Flor. Tot de volgende coffea-break, als ik nog eens in de streek vertoef.
Annemarie, vanaf het laatste jaar diergeneeskunde zijn we veel met elkaar opgescheept geweest en sindsdien ben je een goede vriendin! Onze skitrip (samen met Gwen) en tripke naar Wenen zullen me altijd bijblijven ☺! Ik wens u het allerbeste met Sam en jullie meisjes Lieke en Sanne. Tot de volgende koffiepauze!

Gert & Sarah, Annemarie & Sam, Wouter & Kara, Alexandra & Thomas, Eva & Dagmar, Gwendolyn & Mark, Nancy, Catherine, Sven & Koen, bedankt voor de regelmatige etentjes, BBQ's en city-tripkes naar Wales (waar we iedere keer onze "stoten uit de studententijd/kliniek" terug kunnen oprakelen en de laatste nieuwtjes uitwisselen). Dat we nog eens met zijn allen in Wales verzeild geraken ☺!

Els, waar is den tijd samen shotjes drinken op dinsdag en daarna naar de Cuba Libre… ge zijt een pracht van een vriendin en kwens u het allerbeste met Dominique en de praktijk (ook nog eens bedankt om en fantastische dierenarts te zijn voor onze hond Zohra).

Emily, bedankt voor de lunch-dates en bijhorende fijne babbels! Ik hoop dat je uw draai wat vindt op het ILVO en veel groetjes aan Emma-Louise!

Eva Dejonghe, ook nen dikke merci voor de leute tijdens de studententijd en het plezier op de congressen waar we reeds samen op beland zijn. Doe da nog goed bij Boehringer ☺. Het allerbeste met Pieter-Jan en uw dochtertjes Roos en Fien.

Lars, bedankt voor de toffe babbels (waarin dak meestal een beetje moest zagen ☺) en om me te laten kennis maken met Ubound! Doe da nog goed bij het M-team en ik kijk al uit naar ons volgende frietjes-eten moment!

Jurgen, bedankt voor de toffe sportmomenten en leuke babbels achteraf.

Caroline, ge waart de (on)gelukkige om mij als promotor te krijgen voor uw thesis. Gelukkig is dat goed afgelopen ☺ (denk ik toch). We hebben ons toch vree geamuseerd als er bloed moest worden getrokken van zeugen ☺. Ik heb u toen "jammergenoeg" niet kunnen overtuigen om in het onderzoek te stappen ☺, maar ondertussen zijt ge een grote naam aan het worden in de varkenswereld (zie trip Madrid…☺). Doe da goe en ik kijk al uit naar onze volgende culinaire ontmoeting!

Julie, Sarah, Jantina, Eva N, Iris VD en Valerie. Bedankt voor het jaarlijkse nieuwjaarsfeestje aka Paasfeestje aka koffie-en-taart-moment en voor de leuke babbels, als we elkaar eens "toevallig" tegen kwamen op de faculteit. Tot onze volgende meeting!

Emma en Jantina (de vroegere patho), allereerst bedankt om de lessen proefdierkunde op te fleuren. Verder ook bedankt voor het toenmalig wekelijkse broodje samen! Ons hartje werd iedere keer gelucht! En als da nog niet genoeg was, werd de rest afgesport! Emma bedankt voor de fijne sportmomenten en de toffe babbels achteraf! Allebei veel succes in alles wat je doet en nog van plan bent.

Charlotte, Hans, Marieke, Filip en Annick, bedankt voor de aangename momenten en leuke middagpauzes in Torhout! Tot dak nog eens wa bloed/biggetjes binnenbreng ☺. Hans, ge zijt nen opperbeste kerel. Da we elkaar in de toekomst wa vaker mogen zien! Doe da nog goed bij DGZ en groetjes aan Mirjan en Fran.

Tom, Kris, Julie, Dirk, Christien en Ilse: toevallig ben ik in jullie groepke binnengerold en ik heb het me nog geen moment beklaagd ☺! Bedankt voor alle leuke momenten (Zweden, New York, Praag, Comedy-avonden, film-avondjes, concerten, feestjes, BBQ's, Game-of-Thrones marathons en weekendjes) die we reeds hebben gehad en nog zullen meemaken. Als ik aan ons ardennenweekendje denk, dan komt mijn glimlach spontaan terug ☺….. En als ik denk aan de GOT-marathon S3 afl9: ben er nog niet goed van… Kijk al uit naar onze volgende meeting!

Vanja, Inge, Els, Lotte, Jeroen en Piet: na onze Canada trip (we want it all!!) zijn we blijven afspreken, wat leidde tot toffe feestjes en lekkere etentjes. Bedankt daarvoor!
Vanja en Inge, ook ne dikke merci voor de memorabele city-trips (Nice, Istanbul en Reims)! De reis naar de Filipijnen deze paasvakantie was weer een schot in de roos, 't was echt de max! 'T was echt zalig hé ☺. Tot de volgende meeting in Boedapest ☺!

Filipa, our other Portugese lady (Miss Portugal 2013), it's a pleasure to know you. Thx for introducing eating sushi and all the funny going-out moments ☺! I wish you all the best with your research.
Delphine, ge zijt een straffe madam! Nen dikke merci voor alle leuke momenten, voor alle steun en ik wens u een fantastische start in uw nieuw huis!

Natuurlijk mag ik mijn huidige collega's bij voeders Ostyn niet vergeten:
Marnix, Véronique, Delphine, Lies en Kevin, ik ben superblij dat jullie in het team zitten ☺.
Marnix, bedankt om me van alles te leren over de varkenswereld!
Véronique, je bent echt een wandelende "Wiki-varkenspedia", dus ook bedankt om me van alles te leren over de varkenswereld, voor het vertrouwen en de leuke babbels.
Delphine, bedankt om me in het begin op sleeptouw te nemen en alle ditjes en datjes

aangaande de job aan te leren.
Lies en Kevin, jullie verdienen echt de Osty-pluim 2013 ☺, daar ik dit jaar toch een tamelijk lange periode ben uitgevallen en jullie dit "zonder morren" (hoop ik ☺) hebben opgevangen! Nog eens nen hele hele dikke merci daarvoor! En dat we als collega's nog veel leute mogen beleven!
Inge, Lieslot, Frederik, Stijn, Krista (2x), Martine, Wouter, Filip, Caroline en al de rest (teveel om op te noemen) bij voeders Ostyn: mercikes voor de aangename samenwerking!

Uiteraard heeft ook mijn familie een belangrijke rol gespeeld tijdens de beleving van mijn doctoraat!
Nonkel Frans & tante Marleen, Charlotte & Dimitar, Frederick & Tine, Bedankt voor jullie interesse en steun!
Dirk & Roos, bedankt voor de leuke babbels en jullie oprechte interesse!
Paul & Chris, bedankt voor de leuke babbels en jullie oprechte interesse!
Eva & kevin, we zien elkaar niet veel, maar als het gebeurt is het altijd plezant! Dat we in de toekomst elkaar wat frequenter mogen zien! En ben blij dat Bavo er een speelkameraadje bijkrijgt ☺!

Patrieck, Johan en schoonzusjes Roselien en Charlotte: Nen mens kan zich geen betere broers of schoonzussen wensen. Super bedankt voor alles en om er altijd voor mij te zijn! Woorden zijn niet genoeg…
En natuurlijk kan ik de 2 oogappels van de familie: mijn nichtje Lavinia en neefje Florian niet vergeten. Jullie stralende oogjes, schaterlachjes en deugnieterij hebben me al talloze keren alle kopzorgen doen vergeten ☺!

Pa en Ma, het is onmogelijk in woorden te zeggen hoe belangrijk jullie zijn voor mij. Het was jaren geleden niet vanzelfsprekend dat ik zomaar aan de studies Diergeneeskunde zou beginnen. Jullie hebben me de kans gegeven om te studeren, en jullie hebben er ook telkens voor gezorgd dat ik niets tekort kwam bij het studeren (zes jaar lang zondagavond me komen droppen in Gent, 'echt eten' meegeven, fietsen helpen herstellen, zorgen voor anatomiemateriaal als er anatomie-examens zaten aan te komen, den auto meegeven in het laatste jaar, zakken kaarsjes werden er gebrand als er examens waren (Lourdes kan er niet aan tippen), teveel dingen om op te noemen eigenlijk…). Toen ik na 2 jaar werken als dierenarts aankondigde dat ik ging doctoreren (terug studeren hé pa), sloeg dit waarschijnlijk in als een donderslag bij heldere hemel. Jullie zijn mij echter altijd onvoorwaardelijk blijven steunen en jullie hebben de voorbije jaren zeer sterk meegeleefd met mij en het verloop van mijn onderzoek. Jullie zijn zelfs in het weekend komen helpen bloedtrekken van de varkens (toen er niemand van het labo kon). Kortom, Jullie zijn echte schatten van ouders ☺!
Vandaag zien jullie het resultaat van jullie goede zorgen en niet-aflatende steun! Ik hoop dat ik jullie trots heb gemaakt. Dank u voor alles, Pa en Ma!

Wie ik zeker niet mag vergeten is onze hond Zohra aka "Zotte Doos". Hoeveel keren heb je mij de voorbije (studenten) jaren niet "geassisteerd" (=meestal liggen slapen ad voeten, naast de stoel, op de stoel naast mij, deels op mijn schoot…) tijdens het studeren-werken-schrijven. Uw "puppyblik" (en dat kan ze nog altijd na 10 jaar) kan wonderen verrichten en het klagen/zagen/panikeren in 1-2-3 doen verdwijnen.

En dan nog 1 ietske op het einde van dit boekske:
Eind mei viel de grond “letterlijk” onder mijn voeten weg na het krijgen van slecht nieuws! Ik wil iedereen via deze manier nog eens bedanken voor alle steun en opbeurmomenten onder gelijk welke vorm de laatste maanden (sms, telefoontjes, kaartjes, mailtjes, (ziekenhuis)bezoek, komen koken, ietske gaan drinken, …). Dat deed/doet echt deugd! Nog eens: nen DIKKE merci!! Ondertussen zijn we een 5-tal maanden verder en ziet de toekomst er gelukkig toch rooskleurig uit ☺!

Tot de volgende!

Marc

All of a sudden, you find yourself somewhere you never expected to be
and it’s nice or it takes some getting used to.
Still, you know you’ll find yourself appreciated somewhere down the line…
So, you go to sleep each night, thinking about tomorrow,
going over your plans, preparing the lists
and hoping that, whatever accidents coming your way, will be happy ones…☺

M. Grey