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Development of a new inactivated porcine reproductive and respiratory syndrome virus (PRRSV) vaccine

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LIST OF ABBREVIATIONS

Aa	Amino acid
Ab	Antibody
AEC	3-amino-9-ethylcarcazole
AT-2	Aldrithiol-2
BAL	Bronchoalveolar lavage
BEI	Binary ethyleneimine
CHO	Chinese hamster ovary
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DEN-2	Dengue-2
DHBV	Duck hepatitis B virus
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
E	Envelope protein
EAV	Equine arteritis virus
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immuno sorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMDV	Foot-and-mouth disease virus
FW	Forward primer
Gal	Galactose
GlcA	Glucuronic acid
GlcNac	N-acetylglucosamine
GP	Glycoprotein
HIV	Human immunodeficiency virus
HNV	Hematopoietic necrosis virus
hpi	Hours post inoculation

HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HS	Heparan sulphate
HSV	Herpes simplex virus
IdoA	Iduronic acid
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPMA	Immunoperoxidase monolayer assay
kb	Kilobase
kDa	Kilodalton
kGy	Kilogray
KV	Killed virus
LDV	Lactate dehydrogenase-elevating virus
LV	Lelystad virus
M	Matrix protein
mAb	Monoclonal antibody
MDCK	Madin-Darby canine kidney
MEM	Minimum Eagle's medium
MeV	Megaelectron volt
MHC	Major histocompatibility complex
MLV	Modified live virus
moi	Multiplicity of infection
mRNA	Messenger RNA
N	Nucleocapsid protein
NDV	Newcastle disease virus
NK	Natural killer
nsp	Non-structural protein
OAS	2'-5'-oligoadenylate synthetase
ORF	Open reading frame
PAM	Porcine alveolar macrophage
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
PK	Porcine kidney
PKR	Protein kinase R
PRCD	Porcine respiratory disease complex
PV	Poliovirus
PRRSV	Porcine reproductive and respiratory syndrome virus
REV	Reversed primer
RHDV	Rabbit hemorrhagic disease virus
RLV	Rauscher leukemia virus
RNA	Ribonucleic acid
RNase L	Ribonuclease L
RRV	Ross river virus
RSV	Respiratory syncytial virus
SARS-CoV	Severe acute respiratory syndrome corona virus
SDS-PAGE	Sodium dodecyl sulfate - poly acrylamide gel electroforese
SHFV	Simian hemorrhagic fever virus
Siglec	Sialic acid immunoglobulin-like lectin
SIV	Simian immunodeficiency virus
Sn	Sialoadhesin
SN	Seroneutralization
SRCR	Scavenger receptor cysteine-rich domain
ss	Single stranded
TCID50	Tissue culture infectious dose with a 50% end point
TNF	Tumor necrosis factor
UV	Ultraviolet
VEEV	Venezuelan equine encephalitis virus
VN	Virus-neutralizing
WB	Western blot
WNV	West Nile virus
WT	Wild type
Xyl	Xylose

Chapter 1

INTRODUCTION

1.1 PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)

1.1.1 HISTORY

Clinical outbreaks of late abortions and early farrowing in sows and respiratory problems in piglets were first reported in 1987 in the United States (Keffaber, 1989). The disease was referred to as "mystery swine disease" until the causative agent, porcine reproductive and respiratory syndrome virus (PRRSV) was identified. The disease spread rapidly through America, Europe and Asia (Dewey et al., 2000; Shimizu et al., 1994; Wensvoort et al., 1991). PRRSV was first isolated in the Netherlands in 1991 and this isolate, Lelystad virus (LV), is considered the European prototype of PRRSV (Wensvoort et al., 1991). Shortly thereafter, PRRSV was also isolated in the United States of America and this American prototype is referred to as VR-2332 (Collins et al., 1992). LV and VR-2332 belong to different genotypes with a low homology (50% to 70% nucleotide identity). Also between PRRSV strains of the same genotype, high genetic variability has been reported (Indik et al., 2000; Kapur et al., 1996; Meng et al., 1995; Stadejek et al., 2002). The relationship between the genome of lactate dehydrogenase-elevating virus (LDV) and the two PRRSV genotypes suggests that PRRSV is derived from LDV (Plagemann, 2003). In the past, the European genotype was only found in Europe and the American genotype in America and Asia (Andreyev et al., 1997; Shibata et al., 1996). Due to reversion to virulence of a VR-2332-derived attenuated vaccine strain, the American genotype is also present in Europe (Botner et al., 1997). Import of pigs from Europe resulted in the introduction of the European genotype into Canada (Dewey et al., 2000). PRRSV is endemic in many, if not all swine-producing countries (Albina, 1997b; Cho and Dee, 2006) and is continuously evolving, resulting in new variants (Goldberg et al., 2003; Rowland et al., 1999b). Vaccination often protects pigs against infection with a homologous strain, but protection against infection with a heterologous strain is not always efficient (Labarque et al., 2004; Meng, 2000; Scortti et al., 2006b; van Woensel et al., 1998). This makes it difficult to develop an efficient vaccine.

1.1.2 CLASSIFICATION

PRRSV is assigned to the family *Arteriviridae* (genus Arterivirus) (Cavanagh, 1997; Conzelmann et al., 1993), together with 3 other members: lactate dehydrogenase-elevating virus (LDV) (mice), equine arteritis virus (EAV) (horses and donkeys), and simian hemorrhagic fever virus (SHFV) (monkeys). Their classification is based on similarities of morphology, genomic organization, replication strategy and protein composition. The family *Arteriviridae* belongs to the order *Nidovirales* (Cavanagh, 1997). The name "Nidovirales" is derived from "nidus" and refers to the "nested" set of 3' co-terminal subgenomic mRNAs from which PRRSV proteins are translated (Snijder and Meulenberg, 1998). Other families that belong to the order Nidovirales are the *Coronaviridae* and *Roniviridae* (Cavanagh, 1997).

1.1.3 STRUCTURE

The positive-sense single-stranded (ss) ribonucleic acid (RNA) genome forms together with a double-layered chain of nucleocapsid proteins (N), a roughly spherical shaped nucleocapsid core of 20 to 30 nm diameter (Benfield et al., 1992; Mardassi et al., 1994; Spilman et al., 2009). The nucleocapsid core is surrounded by a lipid bilayer envelope and forms a spherical virion of 40 to 60 nm (Benfield et al., 1992; Dea et al., 2000; Mardassi et al., 1994; Spilman et al., 2009), containing the 6 viral membrane structural proteins: glycoprotein (GP) 2, envelope protein E, GP3, GP4, GP5 and membrane protein M (Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995a; van Nieuwstadt et al., 1996). The M and GP5 proteins exist as disulfide-linked heterodimers in PRRSV particles (Mardassi et al., 1996; Meulenberg et al., 1995a; Verheije et al., 2002) and GP2, GP3, GP4 and probably E are suggested to form heteromultimeric complexes (Wissink et al., 2005). A schematic representation is given in Figure 1.1.

The PRRS virion stability is determined by factors as temperature and pH (Benfield et al., 1992; Bloemraad et al., 1994). PRRSV can be stored for 5 days at 4°C and for several months at -20°C or -70°C. However, at 37°C to 56°C the infectivity of PRRSV decreases rapidly (Benfield et al., 1992; Bloemraad et al., 1994). PRRSV is stable in cell culture medium with pH 6-7.5, but increasing or decreasing the pH reduces the stability of the virus (Bloemraad et al., 1994).

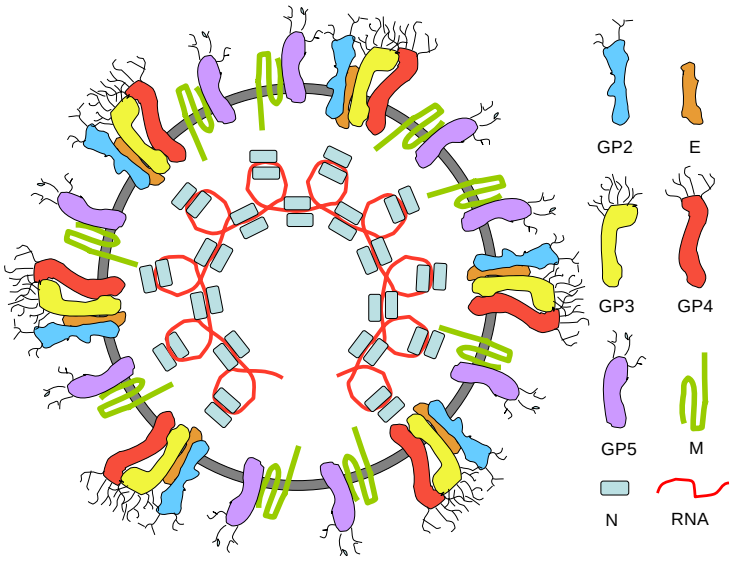


Figure 1.1. A schematic representation of PRRSV. Genomic RNA is associated with N proteins in a core structure, which is surrounded by a lipid envelope containing 6 structural proteins: glycoprotein (GP) 2, envelope protein E, GP3, GP4, GP5 and membrane protein M. The M and GP5 proteins exist as disulfide-linked heterodimers in PRRSV particles and GP2, GP3, GP4 and probably E are suggested to form heteromultimeric complexes (Meulenberg et al., 1995a; Wissink et al., 2005).

1.1.4 GENOME

The genome of PRRSV consists of a polyadenylated positive-sense single-stranded RNA of approximately 15 kilobases (kb) with a 5' cap (Conzelmann et al., 1993; Meulenberg et al., 1997a), and encodes 9 open reading frames (ORFs) flanked by 5' and 3' non-coding regions (Allende et al., 1999; Meulenberg, 2000; Meulenberg et al., 1993a; Snijder and Meulenberg, 1998; Wootton et al., 2000) (Fig. 1.2). The 5' non-coding region carries a cap at its 5' end (Allende et al., 1999), and the 3' non-coding region contains a polyadenylated tail (Allende et al., 1999; Meulenberg et al., 1993b). The expression of the viral genome occurs via the synthesis of a 3' co-terminal nested set of subgenomic mRNAs, which contain a common leader sequence (Conzelmann et al., 1993; Meng et al., 1996; Meulenberg et al., 1993a). The replicase gene is composed of two overlapping ORFs, ORF1a and ORF1b, that are located at the 5' end of the genome, comprising about 75% of the viral genome. ORF1a and ORF1b are processed into at least 12 non-structural proteins (nsp) (Meulenberg, 2000). The structural proteins are encoded by ORF2 to ORF7 and are translated from a co-terminal nested set of subgenomic mRNAs (Meulenberg et al., 1993a; Snijder, 2001). Every subgenomic mRNA contains a 5' common leader sequence, which is derived from the 5' end of the viral genome. Only the 5' ORF of the subgenomic mRNA is translated, except for ORF2 (Meng et al., 1996; Meulenberg et al., 1993a; Snijder and Meulenberg, 1998), which is translated into 2 proteins, GP2 and E.

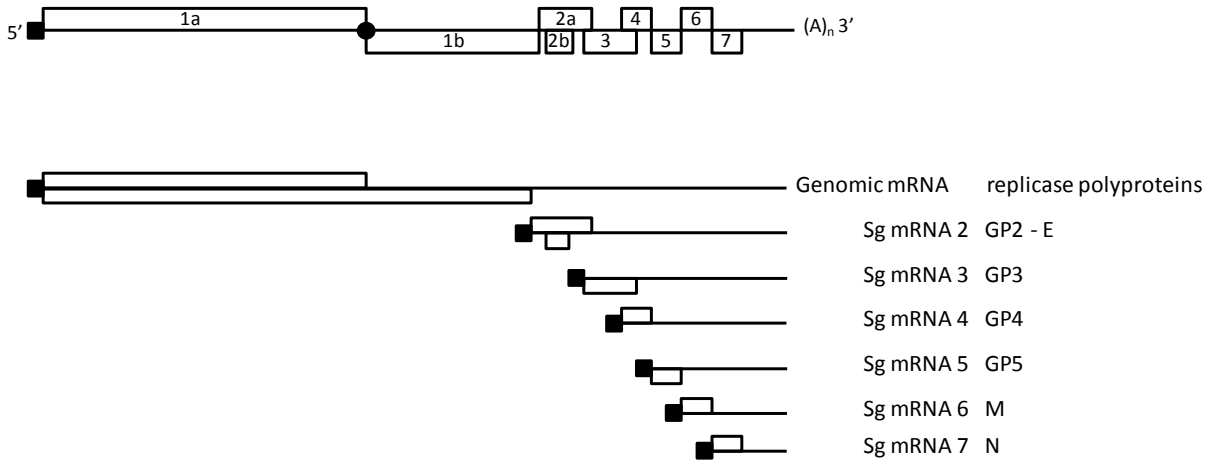


Figure 1.2. PRRSV genome organization (adapted from Meulenberg, 2000). The genome of PRRSV consists of a polyadenylated positive-sense single-stranded RNA of approximately 15 kilobases (kb) and encodes 9 ORFs. ORF1a and 1b encode the non-structural proteins. ORF2 encode the structural proteins GP2 and E. ORF 3, 4, 5, 6 and 7 encode the structural proteins, GP3, GP4, GP5, M and N respectively. The expression of the viral structural proteins occurs via the synthesis of a 3' co-terminal nested set of subgenomic (sg) mRNAs, which contain a 5' common leader sequence

1.1.5 PROTEINS

The PRRSV genome encodes non-structural proteins (nsps) as well as structural proteins. ORF1a and ORF1b represent nearly 75% of the genome and encode the nsps, while ORF2a, 2b, 3, 4, 5, 6 and 7 encode the structural proteins GP2, E, GP3, GP4, GP5, M and N respectively (Mardassi et al., 1995; Meulenberg et al., 1995a; Snijder and Meulenberg, 1998).

Non-structural proteins (nsps)

Nsps are necessary for replication of the virus in the host cell. They are encoded by ORF1a (nsp1 to nsp8) and ORF1b (nsp9 to nsp11). Little is known about the individual functions of the PRRSV nsps, but some functions can be predicted based on observations for EAV. Nsp1 (cysteine protease), nsp2 (cysteine protease) and nsp4 (serine protease) are suggested to have proteolytic capacity to cleave ORF1 proteins into at least 12 nsps (den Boon et al., 1995; Meulenberg, 2000; Snijder and Meulenberg, 1998; Snijder et al., 1995; Snijder et al., 1996). Nsp2 and nsp3 are believed to be involved in double lipid membrane vesicle formation and to modify host cell membranes during the formation of the replication complex (Pedersen et al., 1999; Snijder et al., 2001). Nsp9 is suggested to be an RNA-dependent RNA polymerase (Meulenberg et al., 1993b) and nsp10 is presumably a helicase with nucleoside triphosphate hydrolase and RNA unwinding activities (Bautista et al., 2002).

Major structural proteins

A PRRS virion contains three major structural proteins, which represent approximately 90% of the structural protein content: GP5 (25 kilodalton (kDa)), M (18 kDa) and N (15 kDa) (Bautista et al., 1996; Mardassi et al., 1996; Meulenberg et al., 1995b; Meulenberg et al., 1997a; Nelson et al., 1994; Snijder and Meulenberg, 1998).

GP5, which is encoded by ORF5, has a hydrophobic domain that traverses the membrane, an amino-terminal endoplasmic reticulum (ER) signal peptide and putative glycosylation sites (Meulenberg et al., 1995a). GP5 that is incorporated into virions carries N-linked glycans of the high mannose and the complex type (Mardassi et al., 1996; Meulenberg et al., 1995a). The oligosaccharide on amino-acid 44 or 46 appears to be essential for the production of infectious virus (Ansari et al., 2006; Wissink et al., 2004). GP5 forms, together with M, disulfide-linked heterodimers that are incorporated into PRRSV particles (Mardassi et al., 1996).

The M protein, which is the most conserved structural protein of the arteriviruses, is encoded by ORF6. The hydrophobic character suggests its incorporation to the membrane (Meulenberg et al., 1993b). M does not contain N-linked glycosylation sites or an amino-terminal ER signal peptide (Meulenberg et al., 1993b). It is however suggested that the transmembrane domain contains an internal ER signal peptide, since M is also transported to the ER (Snijder and Meulenberg, 2001). The M and GP5 proteins exist as disulfide-linked heterodimers in PRRSV particles (Mardassi et al., 1996). Those M-GP5 complexes are essential for particle formation (Wissink et al., 2005) and are involved in the attachment to the cellular receptor heparan sulphate (Delputte et al., 2002). M also occurs as homodimers, but these are not incorporated into virions (Mardassi et al., 1996). Besides a ligand function to heparan sulphate, the M protein may play a role in virus assembly and budding (Mardassi et al., 1996).

The N protein, encoded by ORF7, is a small, basic and non-glycosylated protein, which is the most abundant protein comprising 40% of the proteins in the virion (Bautista et al., 1996; Snijder and Meulenberg, 1998). N proteins are predominantly present as disulfide-linked homodimers (Mardassi et al., 1996; Meulenberg and Petersen-den Besten, 1996) and form a roughly spherical shaped core structure consisting of a double-layered chain of N proteins around the RNA genome (Spilman et al., 2009). N does not contain an ER signal peptide and is consequently synthesized in the cytoplasm of infected cells (Mardassi et al., 1996). A nucleolar localization signal in N is responsible for the translocation of N into the nucleoli of infected cells (Lee et al., 2006; Rowland et al., 1999a; Rowland and Yoo, 2003). The presence

of N in the nucleoli suggests a role in modulation of translation in the cell by interfering with ribosomal RNA synthesis or ribosome assembly (Lee et al., 2006; Rowland et al., 1999a; Rowland and Yoo, 2003). The highly basic character of N suggests an interaction with the negatively charged RNA during assembly (Mardassi et al., 1994; Meulenberg et al., 1993b; Meulenberg et al., 1995a).

Minor structural proteins

GP2 (29-30 kDa), E (10 kDa), GP3 (45-50 kDa) and GP4 (31-35 kDa) are minor structural envelope proteins (Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995a; van Nieuwstadt et al., 1996).

GP2, which is encoded by ORF2a, has a hydrophobic character. It contains two putative N-linked glycosylation sites (Meng et al., 1995; Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995a) that are occupied by complex type N-glycans. These glycans are not essential for virus particle formation or virus infectivity (Meulenberg and Petersen-den Besten, 1996; Wissink et al., 2004). GP2 has a cleavable amino-terminal ER signal peptide and a transmembrane domain (Meulenberg et al., 1995a). GP2 is suggested to form together with GP3, GP4 and probably E heteromultimeric complexes, which are incorporated into the virus particle (Wissink et al., 2005). A fraction of GP2 is suggested to be folded on itself via disulfide bridges without formation of homodimers or heteromultimers with other viral proteins (Meulenberg and Petersen-den Besten, 1996). GP2 interact, together with GP4, with the receptor CD163 and is therefore important for virus attachment and entry in the host cell (Das et al., 2010).

E is encoded by ORF2b and is a non-glycosylated envelope protein (Snijder et al., 1999). It has a hydrophobic domain, which is traversing the membrane (Snijder et al., 1999). The homology between E and viroporins suggests its function as a virus-encoded ion channel to enhance membrane permeability (Snijder et al., 1999).

GP3 is encoded by ORF3. This envelope protein contains seven putative N-linked glycosylation sites and is the most glycosylated PRRSV protein (Meulenberg et al., 1995a). The structural nature of GP3 is still not clear. GP3 of LV is incorporated into the virus particle (van Nieuwstadt et al., 1996) suggesting its structural nature in European type PRRSV. For the American type PRRSV both structural and non-structural GP3 are observed. The American isolate FL12 contains a structural GP3 (de Lima et al., 2009), whereas it is found in the medium as a secreted molecule for the North American strain IAF-Klop (Gonin et al.,

1998; Mardassi et al., 1998). Differences in the RNA sequence of ORF3 apparently have an influence on the membrane anchor signal.

GP4, encoded by ORF4, has a highly hydrophobic character. It contains four putative N-linked glycosylation sites carrying complex type N-linked glycans (Meulenberg et al., 1997b). Further, GP4 contains a variable amino-terminal ER signal peptide and a transmembrane domain (Meulenberg et al., 1993b; Meulenberg et al., 1995a; Meulenberg et al., 1997a). GP4 is mediating interglycoprotein interactions and also interacts with CD163 (Das et al., 2010).

1.1.6 REPLICATION CYCLE

Host cells

Characteristic for PRRSV is its narrow host cell tropism. *In vivo*, the virus infects a subpopulation of tissue macrophages, which express sialoadhesin (Sn) and CD163. They are mainly found in lungs, lymphoid tissues and placenta (Beyer et al., 2000; Duan et al., 1998; Duan et al., 1997a; Karniychuk and Nauwynck, 2009; Labarque et al., 2000; Molitor et al., 1997; Vanderheijden et al., 2003). *In vitro*, efficient PRRSV replication is only observed in porcine alveolar macrophages (Wensvoort et al., 1991), differentiated monocytes, monocyte-derived dendritic cells and bone marrow-derived dendritic cells (Chang et al., 2008; Delputte et al., 2007a; Duan et al., 1997a; Loving et al., 2007; Silva-Campa et al., 2009; Wang et al., 2007). PRRSV can also be cultivated in a few African green monkey kidney derived cell lines, such as Marc-145, which are often used for *in vitro* experiments on PRRSV (Kim et al., 1993).

Macrophage receptors

The known receptors that are involved in PRRSV infection of macrophages are heparan sulphate (HS) (Delputte et al., 2002; Vanderheijden et al., 2001), sialoadhesin (Sn) (Vanderheijden et al., 2003) and CD163 (Calvert et al., 2007; Van Gorp et al., 2008). Figure 1.3 gives a schematic overview of these receptors. HS is a glycosaminoglycan that is characterized by a linear backbone of alternating glucuronic acid and N-acetylglucosamine residues (Adhikari et al., 2008). It is expressed on the surface of cells in all tissues, which makes it an easily accessible primary receptor for virus attachment. HS interacts with the M-GP5 complex of PRRSV (Delputte et al., 2002; Vanderheijden et al., 2001). Sn is a sialic acid immunoglobulin-like lectin (Siglec) comprising 16 C-2 set immunoglobulin domains and a V-set immunoglobulin domain (Crocker et al., 1998). Sialic acid binding is mediated by the N-

terminal V-set domain. This molecular interaction includes a key arginine residue that forms a salt bridge with the carboxylate group of sialic acid (May et al., 1998). The M-GP5 complex also binds to Sn (Van Breedam et al., 2010). CD163 is a cellular protein containing 9 cysteine-rich domains, a transmembrane domain and a cytoplasmatic tail (Gronlund et al., 2000; Ritter et al., 1999). It is a scavenger receptor that belongs to the scavenger receptor cysteine-rich (SRCR) superfamily (Resnick et al., 1994; Ritter et al., 1999). GP2 and GP4 are found to interact with CD163 (Das et al., 2010).

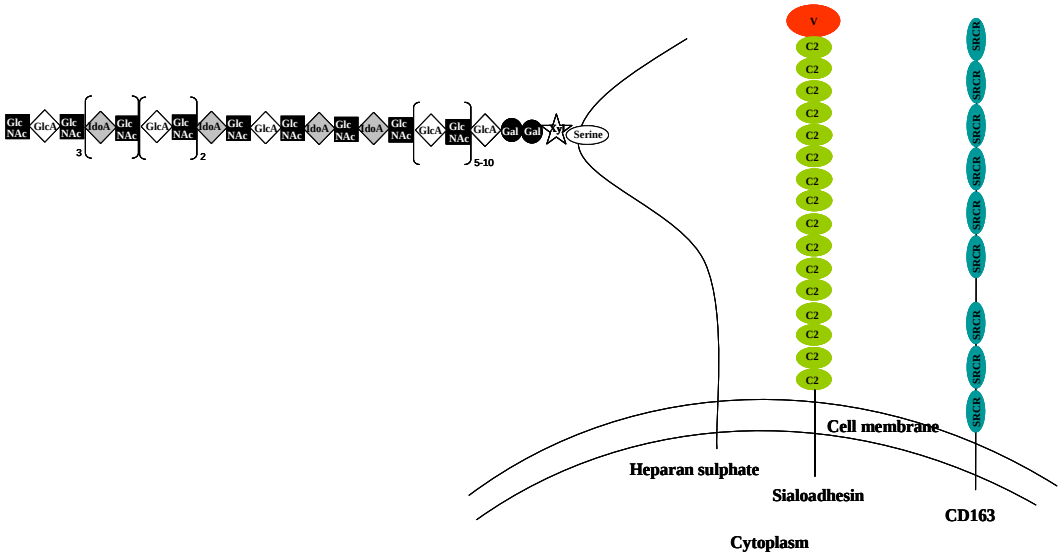


Figure 1.3. Schematic representation of the PRRSV receptors on macrophages: heparan sulphate, sialoadhesin and CD163. ■ = N-acetylglucosamine (GlcNac), ◇ = glucuronic acid (GlcA), ◈ = iduronic acid (IdoA), ● = galactose (Gal), ☆ = xylose (Xyl), SRCR = scavenger receptor cysteine-rich domain.

Marc-145 cell receptors

Receptors that are suggested to be involved in PRRSV infection of Marc-145 cells are a heparin-like molecule (Jusa et al., 1997), vimentin (Kim et al., 2006), CD151 (Shanmukhappa et al., 2007) and CD163 (Calvert et al., 2007). The function of vimentin and CD151 in PRRSV infection still needs to be confirmed. Vimentin is an intermediate-filament protein, which is associated with microfilaments and microtubules. Vimentin is expressed on the cell surface and cytoplasm of Marc-145 cells (Kim et al., 2006). Vimentin is also secreted by activated macrophages (Mor-Vaknin et al., 2003), but a PRRSV receptor function of vimentin was never demonstrated on macrophages. CD151 is a transmembrane glycoprotein and a member of the tetraspanin superfamily. This receptor interacts with the 3' untranslated region RNA of PRRSV (Shanmukhappa et al., 2007).

Replication cycle in macrophages

A schematic representation of a current model of PRRSV replication in macrophages is given in Figure 1.4. PRRSV first attaches to macrophages via HS (Delputte et al., 2002) (Fig. 1.4 (1)). Then, the virus binds to Sn (Vanderheijden et al., 2003) and is internalized through receptor-mediated endocytosis (Kreutz, 1998; Nauwynck et al., 1999) (Fig. 1.4 (2) and (3)). Hereby, the sialic acids present on the viral GP5 interact with the N-terminal sialic acid binding immunoglobulin domain of Sn (Delputte et al., 2004; Delputte et al., 2007b; Van Breedam et al., 2010). After clathrin dependent endocytosis, the virus is transported to an endosome, where a drop in pH is required for virus replication (Nauwynck et al., 1999) (Fig. 1.4 (4)). Also CD163 is involved in infection of macrophages, most probably at the stage of uncoating (Van Gorp et al., 2008). This uncoating assumably requires cathepsin E, an aspartic protease and a not yet identified serine protease (Misinzo et al., 2008). After uncoating of the virus, the genome is released into the cytoplasm for replication. Replication of PRRSV occurs in the cytoplasm of susceptible cells (Benfield et al., 1992). The N protein does not contain an ER signal peptide and as a consequence, it is synthesized in the cytoplasm (Mardassi et al., 1996; Meulenberg et al., 1995a). N proteins interact with newly synthesized viral RNA in the cytoplasm and form viral nucleocapsids. The viral nucleocapsids then go to the ER and/or Golgi complex to form enveloped virus particles (Mardassi et al., 1996). The other structural proteins contain an amino-terminal or internal ER signal peptide and are synthesized and glycosylated in the ER (Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995a) (Fig. 1.4 (5)). Assembly of the mature virus occurs in the lumen of the ER and/or Golgi complex (Snijder and Meulenberg, 1998). The glycans of the glycoproteins of the enveloped particles are finally modified in the Golgi complex (Mardassi et al., 1996; Meulenberg et al., 1995a), which is followed by the release of vesicles containing enveloped nucleocapsids via exocytosis (Dea et al., 1995) (Fig. 1.4 (6)).

Replication cycle in macrophages

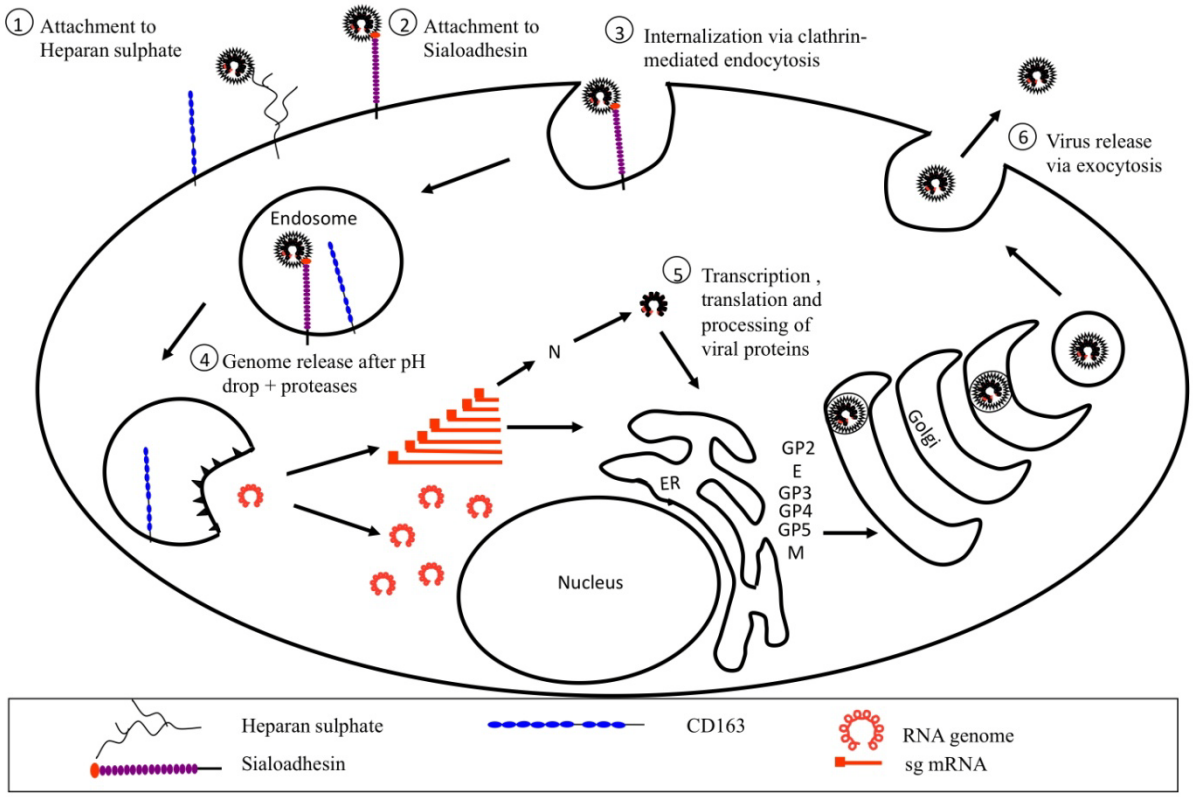


Figure 1.4. A schematic representation of the PRRSV replication cycle in macrophages. First, a virus particle attaches to the host cell (1), followed by receptor-mediated virus entry via clathrin-mediated endocytosis (Nauwynck et al., 1999) (2, 3). Then, the virus particle is transported to an endosome, where it is disassembled. After the viral genome is released in the cytoplasm (4), the nucleocapsid is synthesized in the cytoplasm and forms together with new formed viral RNA a nucleocapsid (Mardassi et al., 1996; Meulenberg et al., 1995a; van Nieuwstadt et al., 1996). The other structural proteins are synthesized in the ER (Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995a; Wissink et al., 2005) (5). After transport through the Golgi complex, new virus particles are released via exocytosis (Mardassi et al., 1996; Meulenberg et al., 1995a; Pol et al., 1997) (6).

Replication cycle in Marc-145 cells

The replication cycle occurs differently in Marc-145 cells compared to macrophages. PRRSV infection of Marc-145 cells is suggested to occur via binding to a heparin-like molecule (Jusa et al., 1997), which resembles the first step of infection of macrophages. Marc-145 cells do not express sialoadhesin, indicating that the virus entry in Marc-145 cells differs from entry in macrophages (Delputte et al., 2004). The nucleocapsid of PRRSV is described to bind to the intermediate filament vimentin expressed on the surface of Marc-145 cells (Kim et al., 2006). Vimentin is also suggested to mediate transport of the virus into the cytosol (Kim et al., 2006). CD151 is suggested to be involved in fusion of the viral envelope and the endosome (Shanmukhappa et al., 2007). Antibodies against CD163 can block PRRSV infection in Marc-145 cells, indicating that CD163 is also essential for PRRSV infection of Marc-145 cells, but its mechanism of action is still unknown (Calvert et al., 2007).

1.1.7 PATHOGENESIS AND CLINICAL SIGNS

Transmission of PRRSV occurs via close contact between an infected and a naïve pig (Albina, 1997a). The infection cycle in pigs initially exposed to PRRSV starts in the macrophages of the respiratory tract, draining lymph nodes, and tonsils, after which the virus enters the blood (Beyer et al., 2000; Pol et al., 1991; Rossow et al., 1996; Rossow et al., 1995). Virus detection starts between 12 hours post inoculation (hpi) and 3 days post inoculation (dpi) in alveolar macrophages, lungs, tonsils, spleen, retropharyngeal lymph nodes, bronchial lymph nodes and thoracic aortic lymph nodes (Duan et al., 1997b). At 14 dpi PRRSV is detected in alveolar macrophages, lungs, tonsils, spleen and bronchial lymph nodes (Duan et al., 1997b). PRRSV can be detected for 5-6 weeks pi in lungs and alveolar macrophages (Christopher-Hennings et al., 1995; Duan et al., 1997b; Rossow et al., 1995). The highest virus titers in blood are detected at 7 dpi to 14 dpi after which it strongly decreases (Duan et al., 1997b; Labarque et al., 2000). The main organs where PRRSV can be found in the acute phase of infection are lungs, lymph nodes, tonsils, spleen and liver (Duan et al., 1997b; Rossow, 1998).

In pregnant sows, PRRSV can cross the placenta, especially during the third trimester of gestation (Lager and Mengeling, 1995; Scortti et al., 2006a). Therefore, PRRSV infection of pregnant sows may result in mummified and weak-born piglets, elevated pre-weaning mortality and late-term abortion (Christianson et al., 1993; Christianson et al., 1992; Lager and Mengeling, 1995; Mengeling et al., 1994; Terpstra et al., 1991). The outcome of reproductive disorders is dependent on the time of gestation when PRRSV infection occurs. PRRSV infection at the onset of gestation has no influence on conception and fertilization (Prieto et al., 1996a; Prieto et al., 1997). Exposure of sows to PRRSV in the early and mid gestation results in a low number of infected fetuses, while infection of sows in the late gestation results in a higher number of death fetuses (Christianson et al., 1993; Christianson et al., 1992; Mengeling et al., 1994). Clinical signs in infected sows or gilts vary from none to anorexia, fever and lethargy (Done and Paton, 1995; Hopper et al., 1992; Mengeling et al., 1994; Terpstra et al., 1991).

In boars, the virus is found in the testes (Christopher-Hennings et al., 1995; Sur et al., 1997; Swenson et al., 1994). PRRSV infection of boars may result in a temporary decrease of sperm quality and shedding of the virus via sperm (Prieto et al., 1996b), which may lead to virus transmission to sows. Clinical signs in boars may consist of fever, loss of appetite and loss of libido, but they are mostly clinically unaffected (Hopper et al., 1992; Nielsen et al., 1997; Prieto et al., 1996b).

Besides its role in reproduction problems, PRRSV is also associated with PRDC. PRRSV infection together with secondary bacterial or viral infections may lead to respiratory problems in pigs of all ages (Rossow et al., 1994; Stevenson et al., 1993). 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).
Finally, PRRSV may persist at lower virus load levels in selected sites of the body, such as lungs, primarily lymphoid tissues and testes, while viremia is absent (Allende et al., 2000; Beyer et al., 2000; Christopher-Hennings et al., 1995; Duan et al., 1997b; Molitor et al., 1997; Murtaugh et al., 2002; Rossow, 1998). The virus is finally cleared from the body after 2 to 4 months pi (Allende et al., 2000).

1.1.8 IMMUNITY

Infection with a pathogen normally induces an immune response that clears the pathogen and protects the host against re-infection. Pigs infected with PRRSV, however, show a prolonged viremia up to 6-7 weeks after infection (Labarque et al., 2000). In some pigs, PRRSV can be isolated for months after initial infection, which suggests a persistent infection (Albina et al., 1994). Thus, PRRSV specific immunity fails to efficiently clear infection.

Innate immunity

In general, the innate immune response upon a PRRSV infection is very weak. Normally, after infection of a cell with a virus, it produces cytokines such as interferon α and β (IFN α/β) (Pfeffer et al., 1998). IFN α/β is responsible for the activation or induction of antiviral proteins such as 2', 5'-oligoadenylate synthetase (OAS), double-stranded RNA-dependent protein kinase R (PKR) and ribonuclease L (RNase L), which are blocking viral replication by degrading RNA and suppressing viral protein synthesis after virus infection (Chebath et al., 1987; Samuel, 2001). PRRSV is susceptible to the antiviral activity of IFN α (Lee et al., 2004; Loving et al., 2007). It is also shown that treatment of macrophages with IFN α *in vitro* increased the sialoadhesin expression on macrophages making them more susceptible to PRRSV infection (Delputte et al., 2007a). Upon infection with PRRSV, the level of IFN α/β production is very low in lungs of the infected pigs (Albina et al., 1998a; Genini et al., 2008; Van Reeth et al., 1999). Also *in vitro*, no significant levels of IFN α/β are produced by macrophages or Marc-145 cells that are inoculated with PRRSV (Albina et al., 1998a; Miller

et al., 2004) (Fig. 1.5). There are several studies indicating that PRRSV replication suppresses IFN α/β production, but the mechanism is not clear (Albina et al., 1998a; Lee et al., 2004; Loving et al., 2007). Accelerated RNA degradation or reduced levels of protein synthesis are possibilities, but are not proven yet. Thus, IFN α plays a dual role during PRRSV infection. On one hand, IFN α can make macrophages more susceptible to PRRSV infection. On the other hand, IFN α can be suppressed by PRRSV infection.

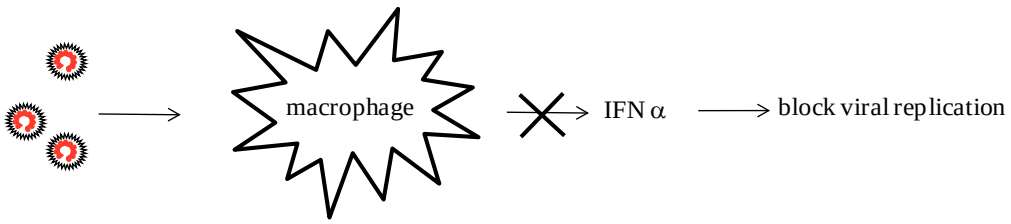


Figure 1.5. IFN α production of macrophages upon PRRSV infection.

In addition to IFN α/β production, inflammatory cytokines are also important in the initial response to a variety of viral respiratory infections (van Reeth and Nauwynck, 2000). Upon PRRSV infection, there is no significant inflammatory cytokine expression (Thanawongnuwech et al., 2001; Van Reeth et al., 1999) (Fig. 1.6). The production of tumor necrosis factor (TNF) α is suppressed in macrophages infected with PRRSV (Chiou et al., 2000; Lopez-Fuertes et al., 2000; Van Reeth et al., 1999). TNF α activates leukocytes to clear pathogens and induces the synthesis of interleukin 1 (IL1) and IL6 in macrophages (Chiou et al., 2000). The production of IL1 after PRRSV infection is under discussion as some authors found an increase of IL1 production (Labarque et al., 2003a; Van Reeth et al., 1999), whereas others describe a suppression of IL1 production (Lopez-Fuertes et al., 2000). TNF α and IL1 are important activators for NF-κB, which is a transcription factor for genes involved in the innate immune response (Christman et al., 2000). IL6 levels are increased in sera of PRRSV infected pigs (Asai et al., 1999; Feng et al., 2003). IL6 is a cytokine involved in B cell differentiation and activation of T cells (Kishimoto, 1989). IL12 is increased in lung cells of infected pigs (Johnsen et al., 2002; Thanawongnuwech and Thacker, 2003). IL12 is produced by macrophages and is important for the induction of a T helper (Th) 1 and cytotoxic T lymphocyte (CTL) response, which will lead to clearance of the virus (Trinchieri, 1995). The level of IL8 expression, which is a neutrophil chemo-attractant, is not increased upon PRRSV infection (Thanawongnuwech et al., 2001). Immunosuppressive IL10 is also increased in peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage (BAL) lymphocytes after PRRSV infection (Chung and Chae, 2003; Feng et al., 2003; Royaee et al., 2004;

Suradhat and Thanawongnuwech, 2003). IL10 is shown to inhibit activation of macrophages, T cells and natural killer (NK) cells (Moore et al., 1993). Taken together, IL10 may be one of the components responsible for the presence of an increased number of macrophages for PRRSV replication and the persistence of PRRSV (Thanawongnuwech et al., 2001).

Next to the antiviral activities, IFN α/β is responsible for the activation of NK cells and dendritic cells (DC) (Biron, 1998; Fitzgerald-Bocarsly and Feng, 2007) (Fig. 1.6). Activated NK cells have a direct cytotoxic activity towards virus-infected cells and in addition they can produce cytokines such as TNF α and IFN γ, that induce an antiviral state and mediate antiviral cytotoxicity (Biron et al., 1999; Lodoen and Lanier, 2006; Rowland et al., 2001). TNF α and IFN γ can activate macrophages (Heise and Virgin, 1995). NK cell-produced IFN γ promotes a CD4 Th1 type 1 response (Manetti et al., 1993). NK cells are observed in the lungs of pigs infected with PRRSV (Samsom et al., 2000). In addition, IFN γ is found in sera of PRRSV-infected pigs (Wesley et al., 2006). However, depletion of NK cells are shown not to enhance PRRSV infection or to influence the ability to clear the virus in pigs (Lohse et al., 2004). DC contribute to virus-induced NK cell activation by producing IFN α, that can activate NK cells and by production of chemokines, that attract NK cells and T cells (Megjugorac et al., 2004). In addition, DC also produce IFN γ (Fitzgerald-Bocarsly and Feng, 2007; Krug et al., 2004). When DC are activated by IFN α, they upregulate the expression of co-stimulatory molecules and MHC II molecules, that can activate CD4+ and CD8+ cells (Grouard et al., 1997). DC trigger the adaptive immune response, but PRRSV can replicate in these cells *in vitro*, thereby preventing their activation (Loving et al., 2007; Wang et al., 2007). PRRSV infection of immature monocyte-derived DC results in a reduced surface expression of major histocompatibility complex (MHC) I and II and CD80/CD86 (Flores-Mendoza et al., 2008; Wang et al., 2007), while PRRSV infection of bone marrow-derived DC results in an increased surface expression of CD80/CD86 and a reduction of surface expression of MHC I (Chang et al., 2008). PRRSV infected DC produce cytokine IL10 (Chang et al., 2008). Other important cells of the innate immune response system are macrophages and neutrophils. Macrophages phagocytose pathogens, thereby triggering the adaptive immunity, by presenting the phagocytosed antigens to T-lymphocytes. Macrophages also produce TNF α (Choi and Chae, 2002), IFN γ (Thanawongnuwech and Thacker, 2003), IL1 and IL10 (Charerntantanakul et al., 2006). The number of macrophages, however, can be decreased due to the ability of PRRSV to induce apoptosis in macrophages (Sur et al., 1998), which may lead to higher susceptibility to secondary infections. Upon PRRSV infection there

is no influx of neutrophils in the lungs, which correlates with the low levels of IL8, a neutrophil chemo attractant (Halbur et al., 1995; Labarque et al., 2000; Van Reeth et al., 1999).

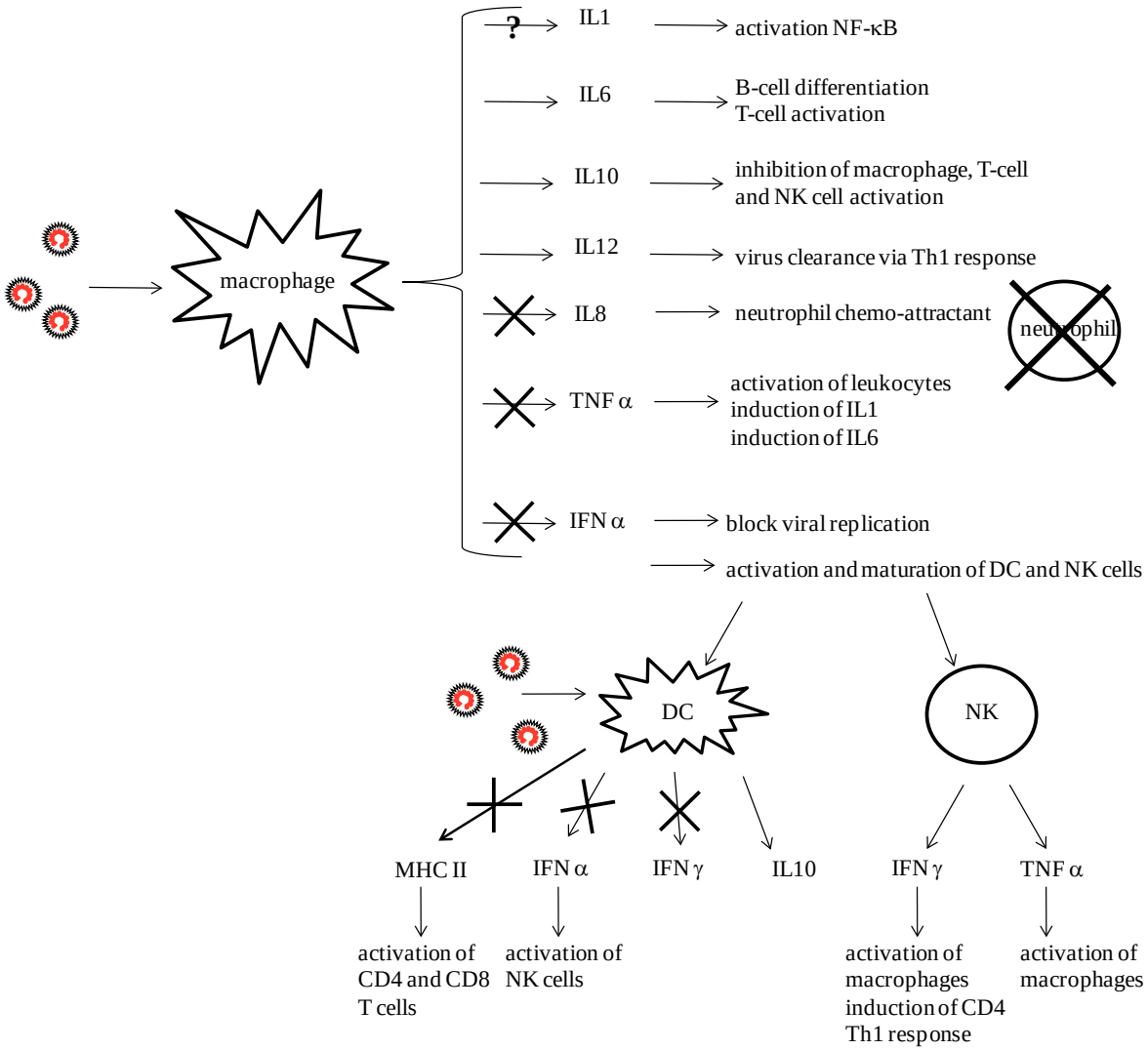


Figure 1.6. Regulation of innate immunity upon PRRSV infection

Adaptive immunity

The adaptive immunity consists of a humoral immune response by specific antibodies and a cell-mediated response by T helper cells and cytotoxic T cells.

For the induction of a humoral immune response, naïve B-lymphocytes capture the viral antigen, process it and present it to CD4+ T helper 2 lymphocytes, which further activate the B-lymphocytes. The activated B-lymphocyte proliferate and differentiate into plasma cells that secrete virus-specific antibodies. Antibodies against PRRSV are detected at 7 dpi (Yoon et al., 1995). Anti-PRRSV immunoglobulin (Ig) M antibodies are observed at 7 dpi, with a peak at 14 dpi (Labarque et al., 2000). After 14 dpi the IgM titers decrease and disappear at 42

dpi (Joo et al., 1997; Loemba et al., 1996). The anti-PRRSV IgA response starts at 14 dpi and increases till it reaches a plateau at 25 dpi, which continues till 35 dpi (Labarque et al., 2000; Murtaugh et al., 2002). IgG antibodies are first detected starting from 7-10 dpi (Joo et al., 1997; Labarque et al., 2000), reach a maximum at 21-49 dpi (Loemba et al., 1996; Vezina et al., 1996), remain constant for several months and decrease at 300 dpi (Loemba et al., 1996; Nelson et al., 1994).

Antibodies against N and nsp2 are detected from the first week pi and are highly antigenic (Dea et al., 2000; Drew, 1995; Nelson et al., 1994; Mulupuri et al., 2008). None of the antibodies in early infection are found to be associated with virus neutralization (Mardassi et al., 1994; Nelson et al., 1994; Plana-Duran et al., 1997). Two weeks after PRRSV infection, antibodies against M and GP5 are induced, although this response is more variable (Loemba et al., 1996; Nelson et al., 1994; Yoon et al., 1995). Only late in infection, neutralizing antibodies appear (Albina et al., 1998b; Diaz et al., 2005; Labarque et al., 2000; Yoon et al., 1994). Neutralizing epitopes are detected on M, GP5, GP4 and GP3, but the *in vivo* relevance in pigs needs to be elucidated (Meulenberg et al., 1997a; Ostrowski et al., 2002; Pirzadeh and Dea, 1997; Weiland et al., 1999; Yang et al., 2000). GP5 contains a neutralizing epitope, which is considered the most important *in vivo* for IAF-Klop (Gonin et al., 1999; Pirzadeh and Dea, 1997). The importance of GP5 in virus neutralization of PRRSV is under discussion. Some authors show that both *in vitro* and *in vivo* virus neutralization is associated with mAbs against GP5 (Gonin et al., 1999; Kwang et al., 1999; Pirzadeh and Dea, 1997; Weiland et al., 1999; Wissink et al., 2003). Others found that the delayed appearance of GP5 antibodies did not correlate with the reduction of viremia, suggesting that GP5 antibodies are not crucial for *in vivo* clearance of the virus (Mulupuri et al., 2008). All glycosylation sites of GP5 are close to the neutralizing epitope and are suggested to hide the neutralizing epitope for neutralizing antibodies and are so reducing virus neutralization (Ansari et al., 2006; Faaberg et al., 2006). Also GP4, M and GP3 contain neutralizing epitopes (Cancel-Tirado et al., 2004; Gonin et al., 1999; Meulenberg et al., 1997a; Weiland et al., 1999). In the amino-terminal region of GP4, an immuno-dominant neutralizing epitope has been identified, but monoclonal antibodies (mAbs) against this epitope neutralize the Dutch isolate I10 less efficient than mAbs against the neutralizing epitope on GP5 (Meulenberg et al., 1997b; Weiland et al., 1999). The role of GP4 in induction of protective antibodies *in vivo* is still unclear. Some studies detected neutralizing antibodies induced by GP4 in pigs (Kwang et al., 1999), while others did not (Gonin et al., 1999). *In vitro,* a neutralizing epitope is found on M (Cancel-Tirado et al., 2004), but its importance in the induction of neutralizing antibodies *in vivo* still needs to be

confirmed. GP3 is suggested to contain neutralizing epitopes (Cancel-Tirado et al., 2004), which appear to induce a protective immune response in pigs (Plana-Duran et al., 1997). Nsp1 and nsp2 are also able to induce a robust antibody response in infected pigs (Johnson et al., 2007; Oleksiewicz et al., 2001). Antibodies against nsp1 are mainly directed against a conformational epitope, while antibodies against nsp2 are directed against linear and conformational epitopes (Johnson et al., 2007; Oleksiewicz et al., 2001). The role of neutralizing antibodies is to protect against re-infection and to reduce viremia and viral spread (Albina et al., 1994; Molitor et al., 1997; Ostrowski et al., 2002). However, viremia may still occur in the presence of neutralizing antibodies (Labarque et al., 2000; Rossow et al., 1994; Vezina et al., 1996; Wills et al., 1997). Also the opposite can occur, viremia can be resolved without the presence of neutralizing antibodies (Diaz et al., 2006; Mulupuri et al., 2008). Taken together, neutralizing antibodies are not the only factor to reduce viremia in a PRRSV infection.

Upon virus infection, antigen-presenting cells present the viral antigen via MHC I and II molecules to CD8+ or CD4+ T-lymphocytes, members of the cell-mediated immunity. PRRSV infected DC cells *in vitro*, fail to induce the expression of MHC I and II and CD80/86 co-stimulatory molecules, which may explain the ineffective T lymphocyte activation (Loving et al., 2007; Wang et al., 2007; Flores-Mendoza et al., 2008). Activated CD8+ T-lymphocytes proliferate and differentiate into CD8+ CTL and activated CD4+ T-lymphocytes into CD4+ Th1 and Th2 lymphocytes. The number of total lymphocytes, CD4+ and CD8+ cells decrease in the blood of PRRSV infected pigs after 3 dpi. After 8-10 dpi, the number of CD8+ cells and lymphocytes is restored (Nielsen and Botner, 1997; Shimizu et al., 1996), while the number of CD4+ cells decreases for at least 14 days (Shimizu et al., 1996). The T-cell proliferation response to PRRSV is first detected at 4 weeks pi and is observed until 9 weeks pi (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). An increased number of CD8+ T-lymphocytes has been observed from 10 dpi in spleen, blood, lungs and lymph nodes of PRRSV-infected pigs (Albina et al., 1998b; Kawashima et al., 1999; Lamontagne et al., 2003; Samsom et al., 2000; Shimizu et al., 1996), which suggests that cytotoxic T cells migrate to the place of infection. In PBMC of infected pigs, however, no cytotoxic T-lymphocyte activity could be detected (Costers et al., 2009). The responsive T cells are predominantly CD4+ cells (Lopez Fuertes et al., 1999). If PRRSV induces a CD4+ Th1 or Th2 response is a matter of debate. Lopez Fuertes et al. (1999) suggested a CD4+ Th1 response, because of the induction of IFN γ and IL2 and reduction of IL4 and IL10. Diaz et al. (2005), however detected IL10 production and no differences in IL4 secreting cells between PRRSV-infected

and control pigs. The CD4+ Th1 response is weak after PRRSV infection, as shown by a low frequency of PRRSV-specific IFN γ secreting cells appearing in the blood (Diaz et al., 2005; Meier et al., 2003). T cell responses induced by GP2, GP4, GP5, M and N are observed (Bautista and Molitor, 1997). M is the strongest inducer of proliferation, followed by GP5, GP4, GP2 and N, with N being the weakest inducer (Bautista and Molitor, 1997). The effect of several PRRSV proteins on cell-mediated and humoral adaptive immunity are summarized in Table 1.1.

Table 1.1. The effect of several PRRSV proteins on cell-mediated and humoral adaptive immunity

PRRSV protein	Cell-mediated immune response	Humoral immune response	References
Nsp1 and nsp 2	NT	+	Mulupuri et al., 2008; Johnson et al., 2007; Oleksiewicz et al., 2001
GP2	++	NT	Bautista and Molitor, 1997
GP3	NT	++	Plana-Duran et al., 1997
GP4	+++	++	Bautista and Molitor, 1997; Kwang et al., 1999; Gonin et al., 1999
GP5	++++	+++	Bautista and Molitor, 1997; Gonin et al., 1999; Kwang et al., 1999; Pirzadeh and Dea, 1997; Weiland et al., 1999; Wissink et al., 2003; Mulupuri et al., 2008
M	+++++	+	Bautista and Molitor, 1997; Cancel-Tirado et al., 2004
N	+	-	Bautista and Molitor, 1997; Mardassi et al., 1994; Nelson et al., 1994; Plana-Duran et al., 1997

NT = not tested, - = not sensitive, + = low sensitivity → +++++ = high sensitivity

1.1.9 PRRSV VACCINATION

Two main types of whole virus vaccines are currently used to prevent PRRSV infection: modified live virus (MLV) vaccines and killed virus (KV) vaccines (Christopher-Hennings et al., 1997; Dewey et al., 1999; Labarque et al., 2003b; Meng, 2000; Mengeling et al., 1999; Mengeling et al., 2003; Misinzo et al., 2006; Nielsen et al., 2002; Nielsen et al., 1997; Nilubol et al., 2004; van Woensel et al., 1998; Zuckermann et al., 2007).

Modified live virus (MLV) vaccines

MLV vaccines can induce a protective immune response against PRRSV infection, but there are concerns about safety. An MLV vaccine can still spread to fetuses via the placenta (Dewey et al., 1999; Nielsen et al., 2002), can be shed via semen and reduce semen quality after vaccination (Christopher-Hennings et al., 1997; Nielsen et al., 1997; Nilubol et al., 2004)

and the vaccine virus may revert to virulent virus (Botner et al., 1997; Nielsen et al., 2001; Nielsen et al., 1997). Another problem is the RNA nature of the virus, which results in genetic variation (Forsberg et al., 2002; Meng, 2000). MLV vaccines only protect against infection with viruses related to the vaccine virus (Labarque et al., 2004; Labarque et al., 2003b; Meng, 2000; van Woensel et al., 1998). MLV vaccines have been shown to reduce the virus replication in pigs and the severity and duration of disease after challenge with a homologous strain, but it does not have a strong impact in vaccinated pigs upon challenge with a virulent heterologous strain (Meng, 2000). Pigs vaccinated with an MLV vaccine showed a statistically significant reduction in clinical signs in terms of incidence, duration and severity of infection and by a more efficient cell-mediated immune response in the vaccinated pigs as compared to the unvaccinated controls after a heterologous challenge, but there were no differences in magnitude of viremia between control and vaccinated pigs (Martelli et al., 2009). Vaccination of boars with an MLV vaccine results in a decrease of viremia and shedding of virus in semen upon challenge with a homologous virus strain (Nielsen et al., 1997). Vaccine intervention with an MLV vaccine in boars reduced the duration of viral shedding, but did not reduce the viral load in tissues or the proportion of persistently infected pigs upon challenge with a heterologous virus strain (Cano et al., 2007).

Commonly used MLV vaccines in Belgium are Porcilis (Intervet International, The Netherlands), Ingelvac PRRS MLV, originally called RespPRRS and RespPRRS/Repro (Boehringer Ingelheim) and Amervac-PRRS/A3 (Hipra Lab). Another MLV vaccine used in the US is Prime Pac PRRS vaccine (Schering Plough Animal Health Corporation).

Killed virus (KV) vaccine (= inactivated virus vaccine)

Killed PRRSV vaccines are safe and easier to adapt to circulating PRRSV strains than an attenuated virus vaccine. It is much easier to develop a KV vaccine against multiple virus strains than to develop an attenuated virus vaccine against the same virus strains (Labarque et al., 2004; van Woensel et al., 1998). However, at present, KV vaccines on the market do not protect enough against PRRSV replication (Meier et al., 2003; Nielsen et al., 1997; Nilubol et al., 2004).

Commonly used KV vaccines in Belgium are Progressis (Merial) and Ingelvac KV (Boehringer, Ingelheim). Other KV vaccines are Suvaxyn-PRRS, originally called Cyblue (Fort Dodge veterinaria USA) and PRRomise (Intervet Shering Plough, USA). Vaccinations with Progressis result over time in a significant improvement of sow reproductive performance (e.g. reduction of premature farrowing, abortions and increase of farrowing rate)

and litter characteristics (e.g. increase of the number of live born and weaned pigs and decrease of stillborn, mummified, weak and splay-legged piglets) (Papatsiros et al., 2006). Vaccination with Cyblue does not change the onset, duration or level of viremia or shedding of virus in semen (Nielsen et al., 1997). Nilubol et al. (2004) tested the effect of a killed PRRSV vaccine (PRRomiSe, Intervet, USA) in seropositive pigs. A first observation was that the magnitude and the duration of viremia were not different between vaccinated pigs and control pigs. A second observation was that the SN titers of vaccinated pigs were higher than the control pigs (Nilubol et al., 2004). Preliminary experiments in our lab showed that it is possible to induce neutralizing antibody production with an experimental inactivated vaccine, but could only partly block viremia after challenge (Misinzo et al., 2006).

Experimental vaccines

Also DNA vaccination has been tested. Barfoed et al. (2004) cloned a PRRSV viral protein or combinations of them in plasmid vectors. Mice and pigs were vaccinated with these deoxyribonucleic acid (DNA) constructs. Neutralizing antibodies were detected in all pigs, with ORF5 vaccinated pigs showing the highest titers (Barfoed et al., 2004). Next to plasmid vectors, also viruses, like pseudorabies virus or bacteria, like mycobacterium bovis can be used as a vector to express recombinant PRRSV proteins. Pseudorabies expressing GP5 of PRRSV failed to induce neutralizing antibodies, but partial protection against clinical disease and a reduction in the duration of the viremia upon PRRSV challenge could be observed (Qiu et al., 2005). *Mycobacterium bovis* expressing a truncated form of GP5 and M protein of PRRSV was constructed by Bastos et al. (2004). At 30 dpi, pigs inoculated with this construct developed a specific humoral immune response against the viral proteins and at 60 dpi, three out of five animals developed neutralizing antibodies. Upon challenge with PRRSV, pigs showed lower temperature, viremia and virus load in bronchial lymph nodes than control animals, suggesting partial protection against PRRSV infection (Bastos et al., 2004). Vaccination with recombinant proteins is also possible. Baculovirus expressing GP3 and GP5 of PRRSV were used to infect insect cells. Vaccination with the insect cells expressing recombinant GP3 and GP5 showed partial protection against PRRSV induced reproductive disorders, while recombinant N had no effect upon challenge with PRRSV (Plana Duran et al., 1997).

1.2 OVERVIEW OF VIRUS INACTIVATION METHODS USED FOR THE GENERATION OF INACTIVATED VIRUS VACCINES

This thesis focuses on the development of a new inactivated PRRSV vaccine. Therefore an overview is given of virus inactivation methods used for the generation of an inactivated virus vaccine, also called a killed virus (KV) vaccine.

1.2.1 INTRODUCTION

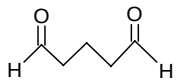
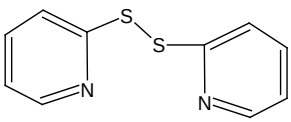
Several problems are associated with KV vaccines. First, virus inactivation can turn out to be incomplete in some cases with outbreaks as a consequence (Beck and Strohmaier, 1987; King et al., 1981; Patil et al., 2002). Second, an enhanced infection after vaccination is also possible, as reported for respiratory syncytial virus (RSV) (Openshaw et al., 2001). The neutralizing epitopes may be destroyed by the inactivation procedure, which results in a poor neutralizing antibody response and a poor protection upon challenge (Cham et al., 2006; Niedrig et al., 1993; Jahrling and Stephenson, 1984). A crucial step in the development of a KV vaccine is a balanced inactivation of the virus. All virus particles need to be completely inactivated, but the inactivation method should have only a minor effect on the antigenic properties of viral components, since the immune system of the host has to recognize the neutralizing epitopes to produce neutralizing antibodies against the antigen.

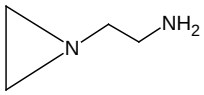
Some inactivation procedures, using cross-linkers or denaturing agents, act on viral proteins (Cheung and Nimni, 1982; Fraenkel-Conrat and Mecham, 1949; Lelie et al., 1987; Schlegel et al., 2001; Tano et al., 2007; Weismiller et al., 1990), and may modify neutralizing epitopes that are essential for induction of neutralizing antibodies. Thus, it would be better to inactivate virus for KV vaccines with methods that do not affect the viral proteins. Other inactivation procedures, using radiation and alkylating agents, mainly have an influence on the genome (Broo et al., 2001; Grieb et al., 2002; Miller and Plagemann, 1974), and will most likely preserve the viral neutralizing epitopes. Beta-propiolactone, a very hazardous product, was used to inactivate viruses, such as rabies virus (Monaco et al., 2006; Mondal et al., 2005) and poliovirus (Jiang et al., 1986). Because of the hazardous nature of this product, more safe products were developed (Bahnemann, 1975; Larghi and Nebel, 1980). N-(2-aminoethyl)ethyleneimine or so-called binary ethyleneimine (BEI) for example, is already often being used for the development of KV vaccines, because it targets only the genome if

low concentrations are used and it is less hazardous compared to beta-propiolactone (Broo et al., 2001; Larghi and Nebel, 1980; Mondal et al., 2005). It has been used for inactivation of DNA viruses, such as African swine fever virus, porcine parvovirus, bovine rhinotracheitis virus and pseudo-rabies virus and for inactivation of RNA viruses, such as foot-and-mouth disease virus, Newcastle disease virus and rabies virus (Bahnemann, 1976; Bahnemann, 1990). Radiation, like ultraviolet (UV) and gamma irradiation is also a promising inactivation method, because this mainly targets the genome and has no or minor effects on the viral proteins. In conclusion, to develop a KV vaccine, the inactivation procedure used, should ensure that the virus is not infectious anymore and that it preserves epitopes necessary for induction of neutralizing antibodies.

In the following paragraphs different inactivation procedures for viruses will be discussed and their usefulness for the development of a PRRSV KV vaccine will be evaluated. An overview of the discussed inactivation methods and their chemical structure are given in Table 1.2.

Table 1.2. Overview of inactivation methods used for the development of KV vaccines

Method	Type	Mechanism
Formaldehyde 	Alkylating agent Cross-linker	Monohydroxymethylation of adenine (Alderson, 1964) Cross-linking of RNA to capsid proteins (Feron et al., 1991; Kuykendall and Bogdanffy, 1992; Ma and Harris, 1988), causing a block of genome reading (Permana and Snapka, 1994) Cross-linking of proteins by formation of inter- and intramolecular methylene bridges between hydroxymethylated amines (Fraenkel-Conrat, 1954)
Glutaraldehyde 	Cross-linker	Cross-linking of proteins by a similar mechanism as formaldehyde described above (Cheung and Nimni, 1982)
AT-2 	Cross-linker	Cross-linking of proteins by oxidation of S-H groups causing formation of S-S bridges which results in covalent modification and functional inactivation of S-H-containing internal viral proteins (Chertova et al., 2003)
pH	Denaturating agent RNA degradation	Denaturation of viral functionally active proteins (Weismiller et al., 1990) The close proximity of the hydroxyl group to the phosphor center of each internucleotide linkage facilitates transesterification under strongly acidic or strongly basic conditions, with a breakage of the phosphodiester bond as a consequence (Li, 1999).
Temperature	Denaturating agent RNA degradation	A high temperature denatures viral functionally active proteins (Lelie et al., 1987; Schlegel et al., 2001) Virus inactivation at 'low' temperature (below 41°C) is considered to be caused by degradation of the nucleic acid (Dimmock, 1967; Fleming, 1971; Laude, 1981)

Gamma irradiation	Radiation	Viruses are inactivated primarily by direct damage, via disruption of the genome (Grieb et al., 2002) Formation of free radicals which damage proteins (Grieb et al., 2002)
UV irradiation	Radiation	Induction of dimer formation between adjacent uracils in RNA (Miller and Plagemann, 1974; Sinha and Hader, 2002). Dimer formation leads to deformation and cleavage of the sugar backbone causing a block of genome reading More slowly, UV also causes structural modifications of the capsid proteins resulting in the formation of large and small photoproducts (Miller and Plagemann, 1974; Subasinghe and Loh, 1972)
BEI 	Alkylating agent	Alkylation of RNA at low concentrations. Most likely genome reading is blocked by alkylation of guanine or adenine by BEI (Broo et al., 2001; Gates et al., 2004) Alkylation of proteins (nucleocapsid) at high concentrations (Broo et al., 2001)

1.2.2 CROSS-LINKERS

Formaldehyde

Formaldehyde, also known as formalin when diluted in water, has an electron deficient central carbon atom and is therefore electrophilic, as illustrated through one of the resonance forms (Fig. 1.7). As a consequence, a nucleophile, such as a non-protonated amino group, can attack the central carbonyl carbon. This chemical reaction is called a nucleophilic addition.

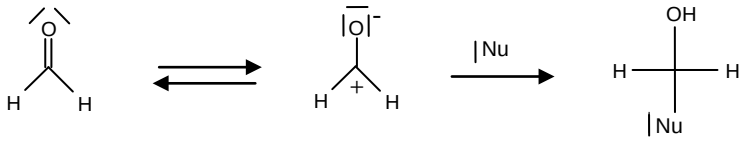


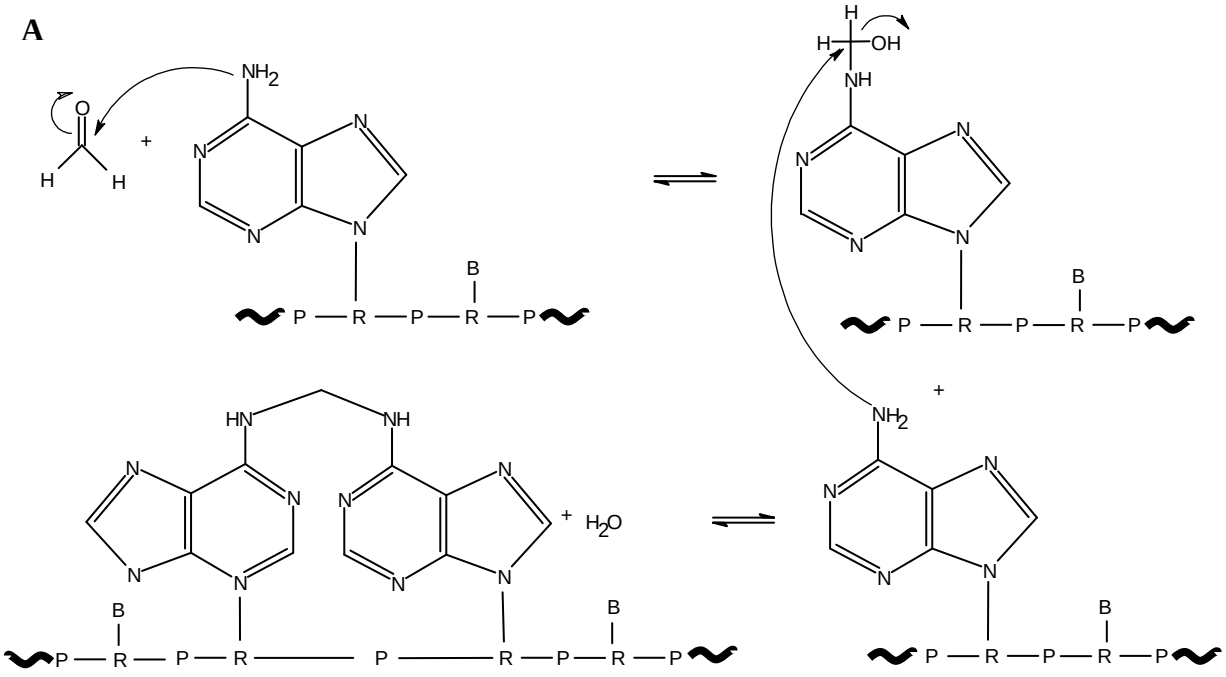
Figure 1.7. Two resonance forms of formaldehyde and a nucleophilic addition reaction

Formaldehyde has an effect on both genome and protein level. First of all, it monohydroxymethylates adenine (Alderson, 1964): the non-protonated exocyclic amine on adenine (N6) acts as a nucleophile and reacts with the central carbon of formaldehyde (Davidson, 1973). Because of this nucleophilic addition, adenine is monohydroxymethylated which causes a block of the genome reading (Fraenkel-Conrat, 1954). This monohydroxymethylation of adenine (Fig. 1.8 A), also known as alkylation, can occur on either DNA or RNA (Fraenkel-Conrat, 1961). Monohydroxymethyladenine (NH – CH_2OH) is stable for days at room temperature and is more likely to exist than the Schiff base (N = CH_2) (Michelson and Grunberg-Manago, 1964). If adenine and formaldehyde are stored for days at

room temperature and at a pH of 4.5, then methylene bis-adenine can be formed (Davidson, 1973).

Secondly, formaldehyde can also react with non-protonated amino groups of the N-terminal amino acid residue and the amino acids containing a nucleophilic nitrogen in their side chain, such as lysine, arginine, glutamine, tryptophan and histidine or a sulfhydryl group in their side chain, such as cysteine: in a first reaction, a non-protonated amine acts as a nucleophile and reacts with formaldehyde. As a result, the amino group is monohydroxymethylated, forming a methylol group as described above for adenine. The loss of a water molecule from the unstable hemi-aminal results in Schiff base formation (Metz et al., 2004), also called an imine intermediate. The resulting Schiff base intermediate can cross-link with arginine and tyrosine and to a lesser extent with glutamine, asparagine, tryptophan and histidine residues by a nucleophilic addition reaction (Metz et al., 2004). In this way inter- and intramolecular methylene bridges can be formed (Fig. 1.8 B) (Fraenkel-Conrat and Mecham, 1949). Because of these bridges, proteins become inter- and intramolecularly cross-linked.

Additionally, these reactions can also cause cross-linking between genome and proteins (Kuykendall and Bogdanffy, 1992; Ma and Harris, 1988), which prevents the transcriptional machinery from reaching the genome (Permana and Snapka, 1994).



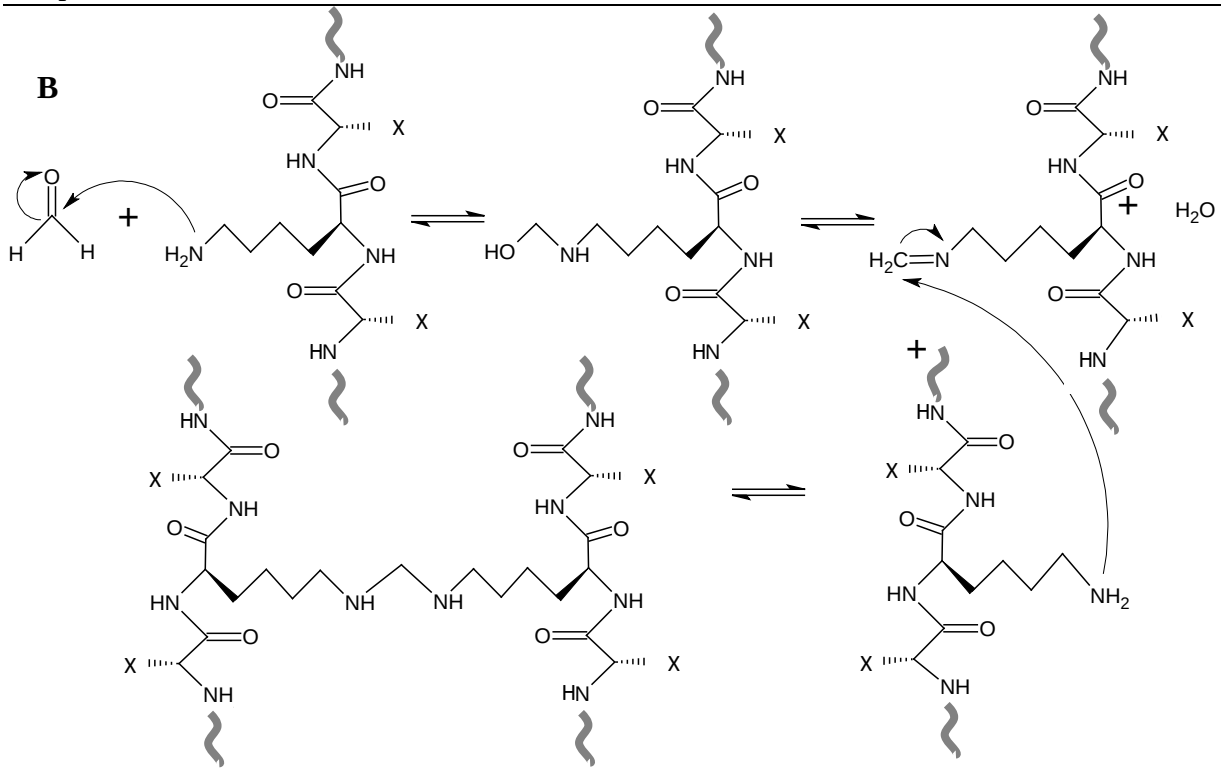


Figure 1.8. Reaction mechanism of formaldehyde with A) DNA or RNA (adenine) and B) amino acids with a primary amine group or sulfhydryl group of proteins: monohydroxymethylation and methylene bridge formation. B = Base (G, T, C, A), R = Ribose, P = Phosphate, X = side chain, = DNA strand, = protein

KV vaccine development based on inactivation with formaldehyde has been investigated for many viruses. A few of them are discussed below and presented in Table 1.3. S ne of them work very well and protect against infection. This accounts for inactivated Ross River virus (RRV) (Kistner et al., 2007), West Nile virus (WNV) (Samina et al., 2005), simian immunodeficiency virus (SIV) (Murphey-Corb et al., 1989) and dengue-2 (DEN-2) vaccines (Putnak et al., 1996). After two vaccinations of mice and guinea pigs with formaldehyde inactivated RRV, no viremia could be detected after challenge (Kistner et al., 2007). The mean level of protection of birds vaccinated with formaldehyde inactivated WNV vaccine in the laboratory was 87%, whereas that of the farm-vaccinated birds was 75%. Although the difference was significant and reflected the farm-to-farm variation in the fitness of the flocks, the level of protection was high enough to result in the complete absence of cases of WN disease since 2001 (Samina et al., 2005). Vaccination with a formaldehyde inactivated SIV vaccine resulted in the protection of eight out of nine rhesus monkeys upon challenge. These results demonstrate that a KV vaccine is highly effective in inducing immune responses that can protect against lentivirus infection and AIDS-like disease (Murphey-Corb et al., 1989). DEN-2 inactivated with formaldehyde retained its antigenicity and was immunogenic in mice and rhesus monkeys. High titers of DEN-2 virus-neutralizing antibodies were observed. Mice

were completely protected against challenge after two vaccinations with 0.15 µg purified inactivated vaccine. Monkeys vaccinated three times with 0.25 µg demonstrated complete absence or a significant reduction in the duration of viremia after challenge with homologous virus (Putnak et al., 1996).

Other vaccines based on formaldehyde inactivation can contain incomplete inactivated virus, which can cause outbreaks of virus infections upon vaccination (Brown, 1993). This is reported for several viruses like foot-and-mouth disease virus (FMDV) (King et al., 1981) and Venezuelan equine encephalitis virus (VEEV) (Brown, 1993). Molecular analysis proved clearly that outbreaks of FMDV in France and Western Europe in the 1980s and VEEV in Central America in 1970s are a consequence of incomplete inactivated virus vaccines (Brown, 1993). Another problem associated with vaccination is atypical or enhanced disease after infection of vaccinated recipients. This is most likely due to the poor induction of neutralizing antibody response together with the formation of immune complexes between virus and antibodies. This is observed with the inactivated measles vaccine (Griffin et al., 2008) and respiratory syncytial virus (RSV) vaccine (Kalina et al., 2004; Openshaw et al., 2001). Some KV vaccines based on formaldehyde inactivation do not protect against challenge, such as killed human immunodeficiency virus (HIV) (Niedrig et al., 1993) and Venezuelan equine encephalitis virus (VEEV) vaccines (Jahrling and Stephenson, 1984). Chimpanzees vaccinated with formaldehyde inactivated HIV induced good humoral and cellular immune responses. However, they were not protected and became infected after challenge (Niedrig et al., 1993). Hamsters received two vaccinations with formaldehyde inactivated VEE vaccine. All hamsters were challenged 45 days after the first vaccine dose. Hamsters vaccinated with the inactivated VEE vaccine were not uniformly protected and all died after challenge with 4.7 $\log_{10}$ plaque-forming unit (PFU) by aerosol route. Twenty-one % died after low-dose (2.5 $\log_{10}$ PFU) aerosol exposure (Jahrling and Stephenson, 1984).

Darnell et al. (2004) examined formaldehyde inactivation of the severe acute respiratory syndrome coronavirus (SARS-CoV) by incubating virus samples with formaldehyde at two different dilutions (1:1000 and 1:4000) and at 4, 25 or 37°C. The formaldehyde exhibited temperature dependence in its ability to inactivate virus. Formaldehyde at a 1:4000 dilution, was not able to completely inactivate virus at 4°C, even after exposure for 3 days. At 25 and 37°C, formaldehyde inactivated most of the virus, close to the limit of detection of the assay, after 1 day. However, some virus still remained infectious on day 3 (Darnell et al., 2004). This suggests that formaldehyde inactivation of SARS-CoV may be an efficient method of inactivation, if proper conditions are met.

Table 1.3. Overview of some vaccines based on formaldehyde inactivation

Virus	Neutralizing Ab	Virological protection	Clinical Protection	Reference
Ross River	NT	Yes	NT	Kistner et al., 2007
West Nile	NT	NT	Yes	Samina et al., 2005
Simian Immunodeficiency	NT	Yes	NT	Murphey-Corb et al., 1989
Dengue	Yes	Partly	NT	Putnak et al., 1996
Foot-and-month disease	NT	NT	No	King et al., 1981
Venezuelan equine encephalitis	NT	NT	No	Brown et al., 1993
Measles	NT	NT	No	Griffin et al., 2008
Respiratory Syncytial	NT	NT	No	Openshaw et al. , 2001
Human Immunodeficiency	NT	No	No	Niedrig et al., 1993
Venezuelan equine encephalitis	NT	NT	No	Jahrling et al., 1984

NT: Not tested

In conclusion, formaldehyde seems to inactivate some, but not all, viruses properly. Problems such as incomplete virus inactivation (outbreaks) have to be kept in mind when using this inactivation method for KV vaccine development. Formaldehyde can be used for KV development for some viruses, but several KV vaccines based on formaldehyde inactivation do not protect the host after challenge with virulent virus. This can be due to modification of viral proteins by formaldehyde as reported for poliovirus (PV), FMDV and HIV (Tano et al., 2007; Patil et al., 2002; Rossio et al., 1998).

Glutaraldehyde

Glutaraldehyde is a saturated 5-carbon dialdehyde. The carbonyl carbons are electrophilic and therefore a nucleophile, such as a non-protonated amino group might attack these carbons in a nucleophilic addition reaction, just like formaldehyde.

Genomic DNA or RNA is a target for glutaraldehyde. Although the exact mechanism of action has not clearly been described, it is likely that glutaraldehyde acts in the same way as formaldehyde, since similar chemical groups are present on formaldehyde and glutaraldehyde (Bedino, 2003). The exocyclic amino group on adenine (N6) is the reactive group, which attacks glutaraldehyde (Hemminki and Suni, 1984). It is certain that glutaraldehyde does have an effect on the genome, since RNA and protein synthesis can be blocked by this aldehyde (McGucken and Woodside, 1973).

Glutaraldehyde can have an effect on proteins as well, because non-protonated amines of amino acids such as lysine can be cross-linked with each other (Cheung and Nimni, 1982). However, the reaction mechanism of glutaraldehyde with proteins is different from that of formaldehyde (Fig. 1.9). First, glutaraldehyde forms unsaturated aldehydes by an aldol condensation and elimination of water (Richards and Knowles, 1968). Then, two amino acids

are linked to glutaraldehyde via a Michael-type addition, a non-protonated amine acts as a nucleophile and reacts with the double bond of the glutaraldehyde polymer (Anfinsen, 1970). When a second non-protonated amine acts as a nucleophile both amino acids are coupled by formation of a bridge (Hermanson, 2008). The intermediate Schiff base or imine would not be formed like in the reaction with formaldehyde (Bedino, 2003; Hermanson, 2008), but both pathways result in covalently linked amino acid side chains.

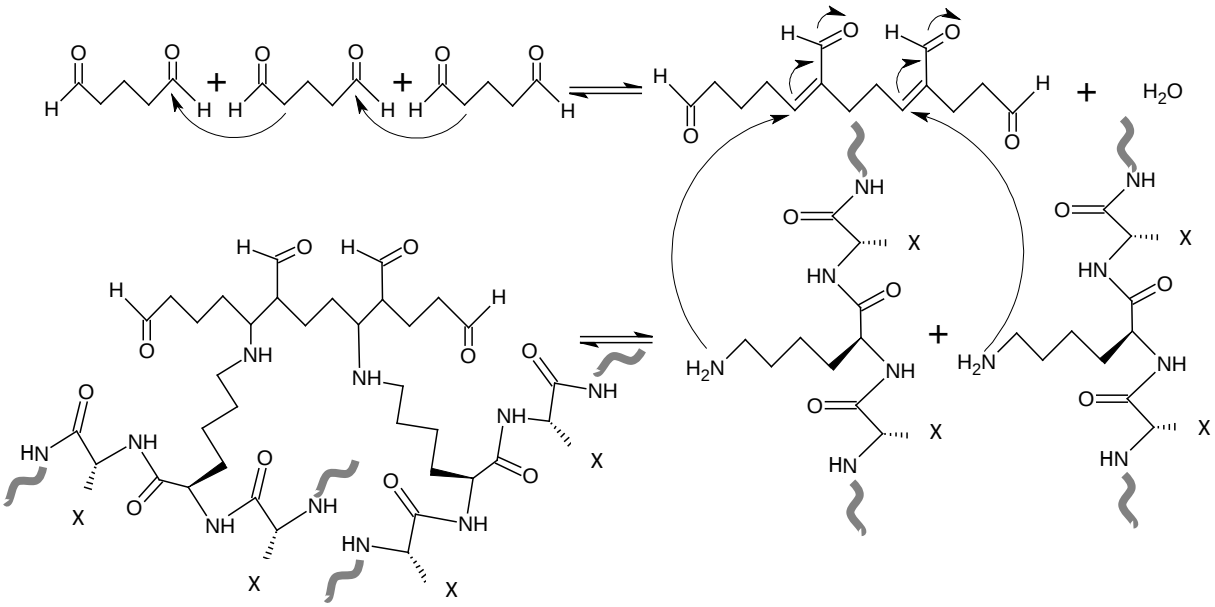


Figure 1.9. Reaction mechanism of glutaraldehyde with amino acids of proteins (e.g. lysine). X = side chain, ~ = protein

Additionally, the reactions described above can also cause cross-linking of the genome and proteins (Kuykendall and Bogdanffy, 1992), which block the genome reading (Permana and Snapka, 1994).

Duck hepatitis B virus (DHBV) is a virus for which the use of glutaraldehyde has been tested for the production of a KV vaccine (Cham et al., 2006). None of the ducks vaccinated with glutaraldehyde inactivated DHBV developed virus-specific antibodies. Four out of four ducks vaccinated with glutaraldehyde inactivated DHBV became infected following challenge. This indicates that there was no induction of protective immunity in these ducks (Cham et al., 2006).

Glutaraldehyde inactivation of SARS-CoV was investigated by incubating virus samples with glutaraldehyde at two different dilutions (1:1000 and 1:4000) and at 4, 25 or 37°C. The glutaraldehyde exhibited temperature dependence in its ability to inactivate SARS-CoV. Glutaraldehyde, at a 1:4000 dilution, was not able to completely inactivate virus at 4°C, even after exposure for 3 days. Glutaraldehyde completely inactivated the virus by day 2 at 25°C

and by day 1 at 37°C (Darnell et al., 2004). This suggests that glutaraldehyde inactivation of SARS-CoV may be an efficient method of inactivation, if proper conditions are met.
In conclusion, glutaraldehyde does not always seem to inactivate viruses properly and if it does, there is not always protection against infection after challenge for the KV vaccines discussed here. This can be due to destruction of neutralizing epitopes by glutaraldehyde.

AT-2 (aldrithiol, dithiodipyridine)

AT-2 oxidizes S-H groups, which results in formation of S-S bridges that cross-link proteins (Fig. 1.10). The internal viral proteins are subjected to the intracellular (reducing) environment, and as a consequence the cysteine residues are present in thiol-form (S-H). In contrast, the surface proteins of viruses are subjected to the extracellular (oxidizing) environment, and as a consequence the cysteines are present as disulfides (S-S). Treatment with AT-2 results in a covalent modification and functional inactivation of S-H-containing internal viral proteins, such as the nucleocapsid protein, that is required for HIV infectivity, whereas the envelope glycoproteins with disulfide bonded cysteines remain unaffected (Chertova et al., 2003). AT-2 treated virions do not retain detectable infectivity, but preserve their conformational and functional integrity.

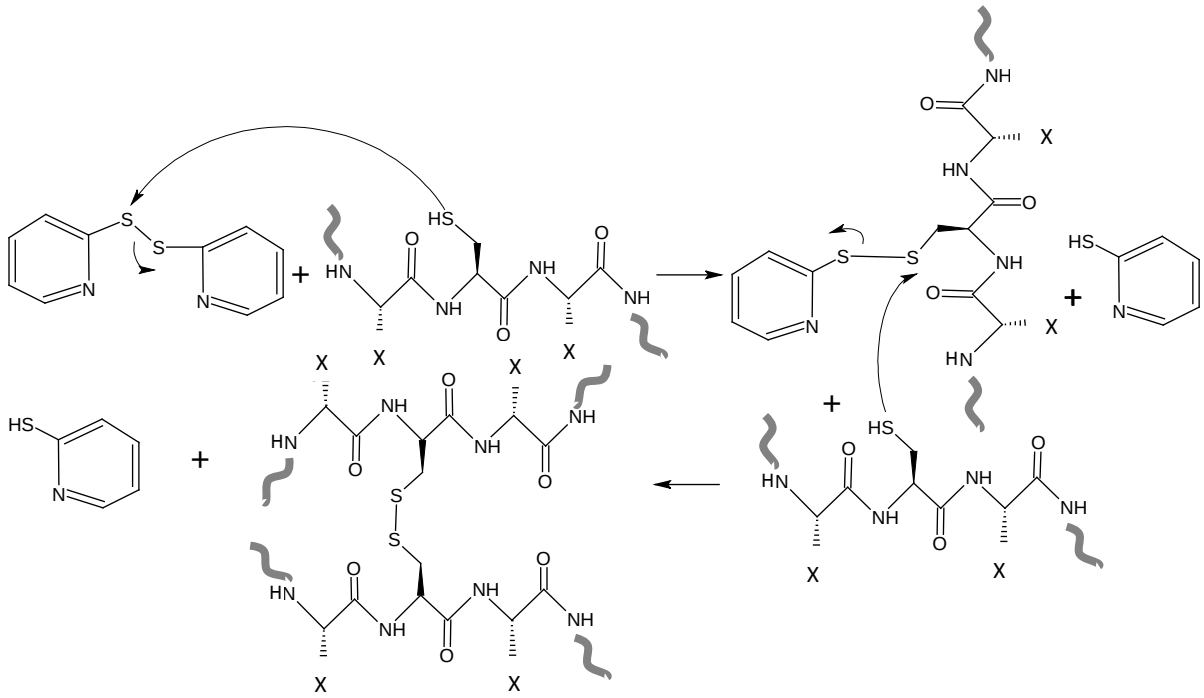


Figure 1.10. Reaction mechanism of AT-2 with cysteine. X = side chain, ~ = protein

This procedure for virus inactivation for KV vaccines has been investigated for HIV (Rossio et al., 1998) and RSV (Boukhvalova et al., 2010). HIV preparations treated with AT-2 showed

maintenance of conformational and functional integrity as demonstrated by high performance liquid chromatography (HPLC) and Western blotting (WB) suggests that such virions may be useful as vaccine antigens (Rossio et al., 1998).

AT-2 inactivated RSV was found to be moderately immunogenic in the cotton rats and did not cause a vaccine-enhancement seen in animals vaccinated with formalin-inactivated RSV. It is shown that compounds that inactivate retroviruses by targeting the zinc finger motif in their nucleocapsid proteins are also effective against RSV (Boukhvalova et al., 2010).

In conclusion, AT-2 seems to inactivate HIV and RSV properly with preservation of the virion structure, but vaccination studies are necessary to prove that AT-2 inactivated virus can protect the host against challenge.

1.2.3 DENATURING AGENTS

pH

Increasing or decreasing the pH has an effect on proteins by protein denaturation. This means that the proteins adopt a different three dimensional structure. A low (acidic) or a high (alkaline) pH can inactivate viruses by denaturation of the secondary structures of proteins, thereby altering the conformation of viral proteins that are involved in attachment to and replication in a host cell. The conformation of spike proteins of coronaviruses for instance changes at a pH of 8 and as a result, entry of the virus into the host cell is initiated, but a lower or higher pH is associated with a loss of reactivity (Weismiller et al., 1990).

The pH also has an effect on the genomic RNA. The close proximity of the hydroxyl group to the phosphor center of each internucleotide linkage facilitates transesterification under strongly acidic or strongly basic conditions (Li, 1999). Base-catalyzed reactions proceed via a nucleophilic addition mechanism where the oxygen attacks the adjacent phosphorus center, with a breakage of the phosphodiester bond as a consequence (Fig. 1.11).

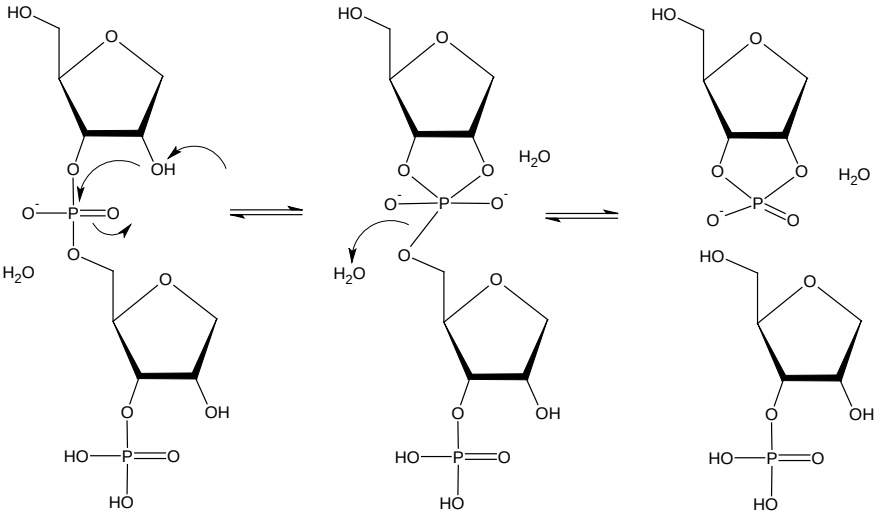


Figure 1.11. RNA degradation in alkaline environment. B = Base (G, U, C, A)

To the best of our knowledge, there are no reports about the *in vivo* use of pH for the inactivation of viruses for KV vaccines. However, inactivation kinetics were performed for SARS-CoV (Darnell et al., 2004). After exposing SARS-CoV to extreme alkaline conditions of pH 12 and 14 for 1 h, and subsequently reversing the conditions to a neutral pH, the virus was completely inactivated. Moderate variations of pH conditions from 5 to 9 had little effect on virus titer, regardless of the temperature. However, highly acidic pH conditions of 1 and 3 completely inactivated the virus at 25 and 37°C. At 4°C, a pH of 3 did not fully inactivate the virus. These data indicate that the infectivity of SARS-CoV is sensitive to pH extremes.
In conclusion, extreme pH can inactivate a virus, but we do not know the effect of a KV vaccine based on this inactivation method in terms of protection upon challenge.

Temperature

By heating or pasteurization of viruses, they can become inactivated by denaturation of the secondary structures of the viral proteins. It is possible that the conformation of the viral proteins involved in attachment to and replication in a host cell are changed in this process (Lelie et al., 1987; Schlegel et al., 2001). Thermal inactivation of viruses by RNA degradation via breakage of the phosphodiester bond is also described (Fleming, 1971) (Fig. 1.11). Virus inactivation at 'low' temperature (below 41°C) is considered to be caused by degradation of the nucleic acid, whereas virus inactivation at 'high' temperature is related to protein denaturation (Dimmock, 1967; Fleming, 1971; Laude, 1981).
Heat inactivation has been studied as a method to develop a KV vaccine for HIV (Poon et al., 2005) and hematopoietic necrosis virus (HNV) (Anderson et al., 2008). HIV samples were

inactivated by three times 10 min incubation at 62°C. BALB/c mice were vaccinated with formaldehyde-stabilized, thermally inactivated virions. Virus-specific antibodies were observed after the first vaccination. In addition, the sera contained antibodies capable of neutralizing the vaccine strain. Poon et al. (2005) also vaccinated rhesus macaques (*Macaca mulatta*), with formaldehyde-stabilized, thermally inactivated virions in the presence of the adjuvant QS 21. Envelope-specific antibodies could be detected after the first vaccination. In 3 out of 3 animals neutralizing antibodies were detectable, but only after the third and final vaccination (Poon et al., 2005).

HNV was inactivated at 50°C for 1.5 hours. The percent mortality of fish in the vaccinated groups was not statistically different compared to the mortality of fish in either the mock-vaccinated or unhandled control groups (Anderson et al., 2008).

To test the ability of heat to inactivate the SARS-CoV, Darnell et al. (2004) incubated the virus at three temperatures (56, 65 and 75°C) for increasing periods of time. They found that at 56°C and 65°C most of the virus was inactivated within a short time. However, a small amount of the virus remained infectious at a level close to the limit of detection for the assay, suggesting that some virus particles were stable at 56°C and 65°C. One possible explanation for this result may be the presence of aggregates that slowly dissociate. While the virus was incompletely inactivated at 56 and 65°C, it was completely inactivated at 75°C after 45 min incubation. Taken together, these results suggest that viral inactivation by pasteurization might be very effective, if the treatment is long enough at a temperature that is high enough.

In conclusion, literature shows that temperature can inactivate virus, but some heat-resistant particles can remain in the inactivated samples. Some heat inactivated vaccines could induce a neutralizing antibody response if the envelope proteins were intact, while others could not prevent infection.

1.2.4 IRRADIATION

Ultraviolet (UV) irradiation

Based on wavelength, UV irradiation is subdivided in three classifications: UVA (320 nm – 400 nm), UVB (280 nm – 320 nm) and UVC (200 nm – 280 nm) (Darnell et al., 2004). UVC is absorbed by RNA and DNA bases, and can lead to formation of dimers between two adjacent pyrimidines (uracil and thymine). UVB can also induce formation of pyrimidine dimers, but 20 to 100 fold less efficient than UVC (Perdiz et al., 2000). UVA is weakly

absorbed by RNA and DNA and forms pyrimidine dimers much less efficient than UVB and UVC (Perdiz et al., 2000).

Thus in general, UV irradiation causes formation of pyrimidine dimers between two adjacent pyrimidines (Miller and Plagemann, 1974; Sinha and Hader, 2002). The two pyrimidines are connected by a pericyclic 2π-2π cycloaddition reaction resulting in the formation of a cyclobutane ring (Fig. 1.12). The dimers cause a certain strain in the sugar backbone of the genome, which possibly leads to breaks in the genome. Additionally, the uracil dimers formed by UV irradiation inactivate the RNA molecule as a transcription template (Sauerbier and Hercules, 1978).

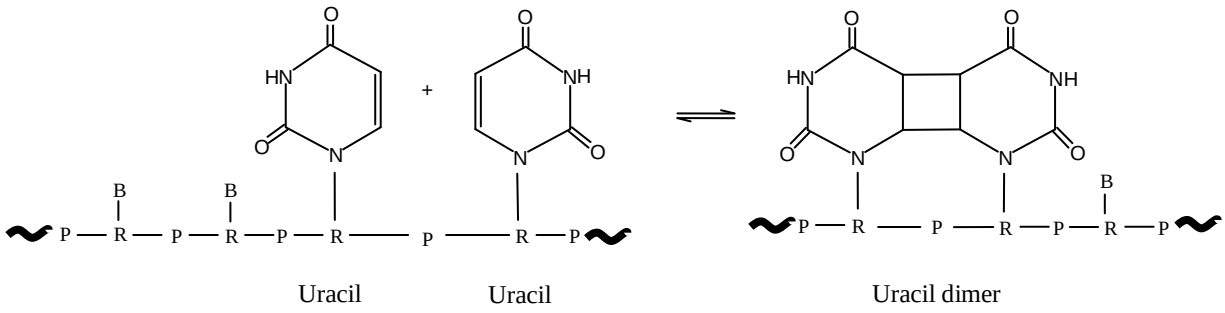


Figure 1.12. Reaction of UV irradiation results in pyrimidine dimer formation . R = ribose, P = phosphate, B = base (G, T, C, A), ~ = DNA strand

The infectivity of mengovirus is lost very rapidly upon exposure to UV irradiation, probably due to dimer formation in the viral RNA (Miller and Plagemann, 1974). Besides this rapid effect on RNA and DNA, UV irradiation also causes structural modifications of the capsid proteins resulting in the formation of large and small photoproducts, which is a much slower process (Miller and Plagemann, 1974). The formation of large photoproduct proteins has also been reported for UV irradiated reovirus (Subasinghe and Loh, 1972).

This procedure for virus inactivation has been tested to develop a KV vaccine against viruses such as rabbit hemorrhagic disease virus (RHDV) (Henning et al., 2005) and Cas-Br-M murine leukemia virus (Sarzotti et al., 1994). Rabbits that were vaccinated with UV inactivated RHDV and challenged with virulent virus did not develop clinical signs of RHD, but all died within 82 hours after challenge. No antibodies were detected at the time of death. These findings show that vaccination with UV-inactivated RHDV does not protect rabbits against challenge with virulent virus (Henning et al., 2005).

On the other hand, UV inactivated the murine leukemia virus called Cas-Br-M (UV-Cas), induced a strong, Cas-specific cytotoxic T lymphocyte (CTL) response in newborn NFS/N mice. The response was detectable for 2 weeks pi and persisted for at least 36 weeks.

Vaccination with UV-Cas protected mice against disease and reduced virus replication (Sarzotti et al., 1994).

In conclusion, literature shows that UV irradiation inactivates some viruses properly. Some KV vaccines based on UV inactivation however do not protect the host after challenge with virulent virus. This might be due to the formation of photoproducts that interfere with the viral proteins.

Gamma irradiation

There are two mechanisms by which gamma irradiation can inactivate biological material. The first mechanism is a direct result of a photon depositing energy into the target. The transfer of this energy results in the dislocation of electrons and breakage of covalent bonds. The second way is indirect damage via free radicals formed after breakage of covalent bonds. Viruses appear to be inactivated primarily by direct damage, via disruption of the genome (Grieb et al., 2002).

This inactivation procedure has been investigated for foot-and-mouth disease virus (FMDV), Rauscher Leukemia virus (RLV) and Herpes simplex virus (HSV) (Smolko and Lombardo, 2005) and Lassa virus (McCormick et al., 1992). FMDV was irradiated with gamma rays and inactivated virus was obtained with a dose of 40 kilogray (kGy), which was used successfully as antigen in the preparation of a vaccine against FMDV. The first commercial lot of this antiviral radiovaccine effectively protected the inoculated cattle against the disease. RLV and HSV were respectively irradiated with gamma rays from a Cobalt 60 source and with a 10 MeV linear electron accelerator. They were inactivated at a dose of 25 kGy (Smolko and Lombardo, 2005).

Rhesus monkeys were vaccinated with gamma irradiation inactivated Lassa virus. The vaccinated animals had a higher antibody titer than the control group. However, after challenge all the monkeys showed viremia and died. The inactivated Lassa virus vaccine based on gamma irradiation could not protect the animals after challenge (McCormick et al., 1992).

In conclusion, gamma irradiation seems to inactivate some viruses properly. Some KV vaccines based on gamma irradiation inactivation protect the host against disease after challenge with virulent virus, while others cannot prevent viremia and disease. However, it is important to realize that free radicals, which destroy viral proteins, may be formed.

1.2.5 ALKYLATING AGENTS

Binary ethyleneimine (BEI) (N-(2-aminoethyl)aziridine, N-(2-aminoethyl)ethyleneimine)

Virus inactivation by ethyleneimines was first introduced more than 30 years ago (Bahnemann, 1976; Warrington et al., 1973). Originally, two chemically similar compounds, aziridine and acetylethyleneimine were proposed for use in development of KV vaccines (Bahnemann, 1975; Bahnemann, 1990). However, the selectivity of aziridine towards nucleic acids is not very high. To improve this selectivity, a product with more protonizable amino groups was necessary, and binary ethyleneimine (BEI), which has two protonizable amino groups while aziridine only has one, was selected.

If a low concentration of BEI is used, the capsid is not alkylated, but BEI passes through the capsid and alkylates the genome (Broo et al., 2001). N7-guanine of the genome acts as a nucleophile and reacts with the electrophile BEI. The nucleophilic substitution reaction performed by N7-guanine causes an opening of the BEI ring and guanine becomes alkylated (Gates et al., 2004) (Fig. 1.13). Also adenine is alkylated via a ring-opening reaction with BEI (Broo et al., 2001). This ring-opening reaction in RNA nucleosides is about 2-3 times faster than in DNA nucleosides (Hendler et al., 1970). To the best of our knowledge, there are no reports on any interaction of BEI with proteins, at least if low concentrations are used, which suggests that the neutralizing viral epitopes are preserved after treatment of a virus with BEI.

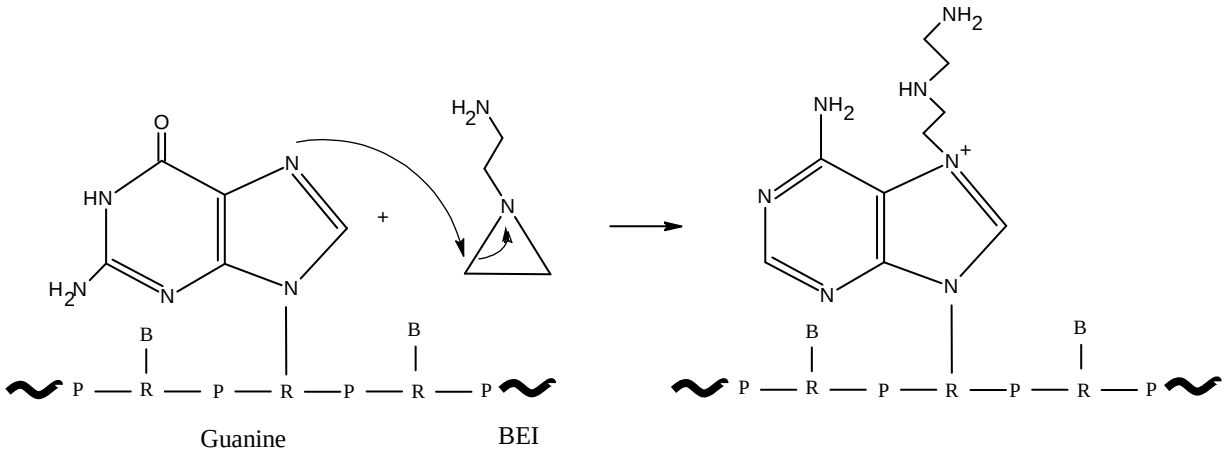


Figure 1.13. Reaction of guanine with BEI. R = ribose, P = phosphate, B = base (G, T, C, A), ~ = DNA strand

BEI is used for the inactivation of many viruses such as foot-and-mouth disease virus (FMDV) (Bahnemann, 1975), Ross River virus (RRV) (Aaskov et al., 1997), sheep pox (Awad et al., 2003), HIV (Race et al., 1995), Newcastle disease virus (NDV) (Buonavoglia et al., 1988) and hematopoietic necrosis virus (HNV) (Anderson et al., 2008).

The inactivation of FMDV with BEI did not affect the antigenicity of FMDV. Although vaccines prepared with FMDV inactivated by BEI were comparable in their immunogenicity to vaccines prepared with ethyleneimine or N-acetylethyleneimine used as inactivants, the first one is much safer to handle (Bahnemann, 1975).

A purified RRV vaccine has been developed by using BEI for virus inactivation. Mice vaccinated with this vaccine were protected against challenge with live virus. The vaccine induced significant levels of neutralizing antibody in all strains of mice tested (Aaskov et al., 1997).

BEI was also used to inactivate a local Egyptian strain of sheep pox virus. Specific antibodies appeared from the first week post vaccination and remained until the fourth week post challenge. The vaccine proved to be safe, sterile and able to induce protection of the vaccinated lambs after challenge with the virulent sheep pox virus up to 6 months post vaccination (Awad et al., 2003).

Race et al. (1995) have shown that the experimental BEI inactivated HIV vaccine induces virus-neutralizing antibodies against both the homologous vaccine strain and a heterologous virus strain (Race et al., 1995).

The activity of a BEI inactivated NDV vaccine was compared to a formaldehyde inactivated NDV vaccine. The BEI inactivated NDV vaccine had almost twice the efficacy (Buonavoglia et al., 1988).

For the inactivation of HNV, 1.5 mM BEI was used. Rainbow trout were vaccinated with BEI inactivated HNV and challenged at 28 or 56 days after vaccination with live HNV. The cumulative percent mortality in the group of fish immunized with the BEI vaccines was not statistically different compared with the mortality of fish in the mock vaccinated control groups when tested 28 or 56 days after vaccination (Anderson et al., 2008).

In conclusion, BEI seems to inactivate viruses properly and most KV vaccines based on BEI inactivation protect the host after challenge with virulent virus, which is expected since BEI does not interfere with the virus structure. Only one KV vaccine did not protect the host upon challenge, but the possible reason remains to be elucidated.

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Chapter 2

AIMS OF THE THESIS

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most important economic viral pig disease worldwide (Neumann et al., 2005). PRRSV infection may result in reproductive failure in sows and is involved in the porcine respiratory disease complex (PRDC) (Christianson et al., 1993; Lager and Mengeling, 1995; Mengeling et al., 1994; Terpstra et al., 1991). To control the disease, several commercial modified live virus (MLV) and killed virus (KV) vaccines are available, but they both currently have disadvantages. MLV vaccines are only effective if the circulating virus strain is closely related to the vaccine strain (Labarque et al., 2004) and MLV vaccines can cause some safety problems such as spreading of vaccine virus and reversion to virulence (Mengeling et al., 1999; Nielsen et al., 2001; Nielsen et al., 1997). Currently available KV vaccines are safe to use, but their efficacy may be questionable (Nielsen et al., 1997; Zuckermann et al., 2007). In theory, an inactivated virus vaccine, also called a KV vaccine may have advantages compared to MLV vaccines, because it is safe for use in pigs without risk of viral spread and reversion (Nielsen et al., 2001). The current inactivated PRRSV vaccines do not protect properly against infection (Nielsen et al., 1997; Zuckermann et al., 2007). This can be due to destruction of neutralizing epitopes during the inactivation procedure or change of the epitopes during adaptation to cell lines. The different processes to develop a vaccine are virus production, virus inactivation or attenuation and vaccine formulation. In this thesis, the optimization of the virus production and the virus inactivation of PRRSV were investigated to develop a KV vaccine.

A first aim was to evaluate PRRSV inactivation methods that allow preservation of the neutralizing epitopes. To have an effective KV vaccine, the virus should be completely inactivated to avoid outbreaks and the neutralizing epitopes have to be preserved in order to induce a proper immune response to be able to clear the virus after an infection. Different inactivation methods, using cross-linkers, denaturing agents, radiation and alkylating agents and their effect on PRRSV were investigated (Chapter 3). Binding and internalization of inactivated PRRSV in macrophages was determined, since entry-associated domains of the PRRS virion are most likely involved in the induction of neutralizing antibodies, because neutralizing antibodies block the entry of PRRSV into macrophages *in vitro* (Delputte et al., 2004). With this quality control assay, it is possible to perform an *in vitro* selection of inactivation procedures that preserve the virus entry-associated domains. KV vaccines based on these selected inactivation procedures were then tested if they were able to induce a neutralizing antibody response *in vivo* (Chapter 3).

A second aim was to develop a PRRSV susceptible cell line expressing macrophage receptors mimicking the natural entry pathway. For the production of PRRSV, a cell line avoiding mutations due to growth on cell lines would be ideal. Virus production for PRRSV vaccine development is currently performed on Marc-145 cells. PRRSV enters Marc-145 cells via a different pathway than in macrophages, the natural host cells (Delputte et al., 2004), which requires adaptation of the virus strains (Collins et al., 1992). Most mutations due to adaptation occur in the non-structural viral proteins, but also mutations in structural proteins are possible. This is not desirable in a KV vaccine, since these mutations can cause modification or loss of neutralizing epitopes. Mimicking the entry pathway in macrophages might avoid mutations due to adaptation for growth on a cell line. To avoid the problems associated with PRRS vaccine virus production in other cell types, non-permissive cells were transfected with sialoadhesin (Sn) and CD163, both important in PRRSV infection of macrophages. The cell lines recombinantly expressed Sn, a receptor that mediates PRRSV attachment to and internalization into macrophages (Delputte et al., 2005; Vanderheijden et al., 2003) and CD163, which is probably involved in virus uncoating in macrophages (Van Gorp et al., 2008). The production of different PRRSV strains on those cell lines was optimized (Chapter 4 part 1). Once the best conditions were found to produce PRRSV on Sn and CD163 expressing cells, a KV vaccine based on virus grown on this cell line was developed. The efficacy of KV vaccines based on PRRSV grown on the cell line and Marc-145 cells were compared (Chapter 4 part 2).

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Chapter 3

DEVELOPMENT OF AN INACTIVATED PRRSV VACCINE USING A RESEARCH ORIENTED PROTOCOL

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3.1.1 SUMMARY

Porcine reproductive and respiratory syndrome virus (PRRSV) causes severe economic losses in the pig industry worldwide. Currently, vaccines based on inactivated PRRSV provide limited protection of pigs against infection, most likely because viral epitopes associated with the induction of neutralizing antibodies are not or poorly conserved during inactivation. To analyze the effect of inactivation procedures on the interaction of PRRSV with receptors involved in virus entry, a new quality control assay was set up in this study. Viral entry-associated domains are most likely important for the induction of neutralizing antibodies, since neutralizing antibodies block interaction of PRRSV with cellular receptors. To investigate the interaction of PRRSV with the cellular receptors upon different inactivation procedures, attachment to and internalization of inactivated PRRSV into macrophages were monitored. AT-2 could not inactivate PRRSV completely and is therefore not useful for vaccine development. PRRSV inactivated with 37°C, ultraviolet (UV) irradiation, binary ethyleneimine (BEI) and gamma irradiation, which all mainly have an effect at the genomic level, showed no difference compared to control live virus at the level of virus entry, whereas PRRSV treated with formaldehyde, glutaraldehyde and pH changes, which all have a modifying effect on proteins, was not able to internalize into macrophages anymore. These results suggest that inactivation with methods with a main effect on the viral genome preserve PRRSV entry-associated domains and are useful for future development of an effective inactivated vaccine against PRRSV. Based on the quality control assay mentioned above, two inactivation procedures were selected to inactivate PRRSV for a vaccination study: UV and BEI. Vaccination with UV- or BEI-inactivated virus induced virus-specific antibodies and strongly primed a virus-neutralizing antibody response. As a consequence a significant reduction in viremia after infection was observed. In contrast, vaccination with a commercial killed PRRSV vaccine did not prime a virus-neutralizing antibody response, resulting in no significant protection against viremia after infection.

3.1.2 INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded enveloped RNA virus which is assigned to the family *Arteriviridae* (Conzelmann et al., 1993; Meulenberg et al., 1993), together with lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). The *Arteriviridae* as well as the *Coronaviridae* and *Roniviridae* belong to the order Nidovirales (Cavanagh, 1997; Cowley et al., 2000).

PRRS is worldwide recognized as the economic most important viral pig disease (Neumann et al., 2005). Infection of pregnant sows may result in mummified and weak-born piglets, elevated pre-weaning mortality and abortion (Christianson et al., 1993; Mengeling et al., 1994; Terpstra et al., 1991). Infected boars may show a temporary decrease of sperm quality and virus shedding via sperm (Prieto et al., 1996). This virus shedding may lead to virus transmission to sows. Besides its effect on reproduction, PRRSV is also involved in the multifactorial respiratory disease complex in pigs, where the virus facilitates secondary bacterial infections and respiratory problems in pigs of all ages (Rossow et al., 1994; Terpstra et al., 1991).

Two main types of vaccines are currently used to prevent PRRSV infection, modified live virus (MLV) vaccines and killed virus (KV) vaccines (Meng, 2000; Zuckermann et al., 2007). Commercial MLV and KV vaccines exist, but both have some disadvantages. MLV vaccines induce an immune response that can protect pigs against PRRSV infection, however only when the virus is not too distant from the vaccine strain (Labarque et al., 2004; Labarque et al., 2003). Attenuated viruses may cause safety problems. Some MLV vaccines may spread transplacentally (Dewey et al., 1999; Mengeling et al., 1999; Nielsen et al., 2002), be shed via semen and reduce semen quality (Christopher-Hennings et al., 1997; Nielsen et al., 1997), and may even revert to virulence (Nielsen et al., 2001). KV vaccines are safe to use, but currently used KV vaccines insufficiently protect pigs against viremia upon challenge, since both magnitude and duration of viremia were not different between vaccinated and control animals (Zuckermann et al., 2007). A comparative study of MLV and KV vaccines in boars showed that while vaccination with an MLV vaccine decreased viremia and virus shedding in semen, vaccination with a KV vaccine did not change onset, duration or level of viremia, or virus shedding in semen (Nielsen et al., 1997). Preliminary experiments in our lab showed that while an experimental inactivated PRRSV vaccine was able to induce neutralizing antibodies, it could only partly block viremia upon challenge (Misinzo et al., 2006). The current

incomplete protection of KV vaccines against PRRSV infection might be caused by an over-inactivation of the virus, resulting in destruction of neutralizing viral epitopes. Neutralizing viral epitopes induce the production of neutralizing antibodies by the host, which are necessary for neutralization of the virus and reduction of overall infection. As a result, destruction of the neutralizing epitopes by over-inactivation will reduce the number of neutralizing antibodies raised by the host, which will ultimately lead to less efficient protection against a viral challenge.

To our knowledge, currently used KV vaccines against PRRSV have been evaluated for the quantity of viral antigens, but not the quality. For other viruses, the quality has been examined. For example, the effect of inactivation of influenza virus is investigated by measuring the hemagglutinating activity before and after inactivation (Di Trani et al., 2003). For HIV, the attachment of neutralizing antibodies to viral epitopes is determined after inactivation by an Enzyme-Linked Immuno Sorbent Assay (ELISA) (Grovit-Ferbas et al., 2000; Poon et al., 2005). ELISA as a tool for quality control of the antigen is also used for rabies virus (Fournier-Caruana et al., 2003; Rooijakkers et al., 1996) and poliovirus vaccines (Morgeaux et al., 2005). Quality control for PRRSV cannot be performed using the same methods as for HIV and influenza, because of the limited knowledge of PRRSV neutralizing epitopes. There are some neutralizing epitopes known on GP5 and GP4 (Meulenberg et al., 1997a; Ostrowski et al., 2002; Wissink et al., 2003) and there are possibly also neutralizing epitopes on GP3 (Cancel-Tirado et al., 2004), but it is not known if other important epitopes exist and which neutralizing epitopes are most important. Previous results from our lab showed that PRRSV neutralizing antibodies block infection by preventing the interaction of PRRSV with the internalization receptor sialoadhesin (Sn) on the target cells, macrophages (Delputte et al., 2005; Delputte et al., 2004; Van Gorp et al., 2008; Vanderheijden et al., 2003). This indicates that neutralizing antibodies are directed to the viral epitopes that are involved in the attachment to the PRRSV receptors and internalization into the macrophage (Delputte et al., 2004). Based on this observation, a quality control of the viral antigen of a PRRSV KV vaccine was developed, by monitoring PRRSV attachment to and internalization into macrophages before and after inactivation. An ideal killed PRRSV vaccine should be able to attach to and internalize into macrophages during a quality-control assay, but it should be disabled to replicate in order to avoid viremia.

The aim of this study is to investigate the effect of different PRRSV inactivation methods on the viral entry-associated domains. To this end, inactivated PRRSV attachment to and internalization into macrophages were monitored. Two inactivation procedures, which

preserved the viral entry-associated domains, were selected to inactivate PRRSV for a vaccination study: UV and BEI. It was investigated if vaccination with UV- or BEI-inactivated PRRSV can induce a protective immune response in PRRSV-negative piglets. The efficacy of these experimental inactivated vaccines was compared with a commercial inactivated PRRSV vaccine.

3.1.3 MATERIALS AND METHODS

Cells and virus

The European PRRSV strain, Lelystad virus (LV) (Wensvoort et al., 1991), grown on Marc-145 cells (fourth passage) was used for inactivation and vaccine preparation. Challenge virus consisted of the fifth passage of LV, propagated in porcine alveolar macrophages (PAM) that were derived from gnotobiotic piglets. Marc-145 cells cultivated in minimum Eagle's medium (MEM) with 5% Fetal Bovine Serum (FBS), 0.3 mg/mL glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.1 mg/mL kanamycin were used for LV production and titration. Virus attachment and internalization were investigated in macrophages cultivated in RPMI 1640 with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mg/mL kanamycin, 0.1 mg/mL gentamycin, 0.01 mg/mL tylosin, 0.3 mg/mL glutamine, 1 mM nonessential amino acids and 1% sodium pyruvate (100x) for 24 hours.

PRRSV concentration and purification

The European PRRSV strain, Lelystad virus (LV), was grown on Marc-145 cells. The medium containing the virus was purified as described by Delputte et al., (Delputte et al., 2004), this with some modifications to allow purification of larger quantities of virus. Virus supernatant was first filtrated through a 0.45 µm filter and then ultra-centrifuged for 2 hours at 31 000 rpm with a rotor type 35 at 4°C (Beckmann Coulter) to pellet the virus. The resuspended virus pellet was centrifuged 10 minutes at 13 000 rpm (Heraeus fresco) to remove cell debris and large aggregates and the supernatant was ultra-centrifuged through a 30% sucrose cushion for 3 hours at 30 000 rpm with a SW 41 Ti rotor at 4°C (Beckmann Coulter). Finally, the virus pellet was resuspended in 1 mL PBS by incubating it for 1 hour on ice.

Inactivation methods

An overview of the inactivation methods used and their mode of action is shown in Table 1.2 in the introduction of the thesis. Purified virus (10^7 $TCID_{50}$/mL) was used for inactivation. For inactivation with formaldehyde, glutaraldehyde or 2,2-dithiodipyridine (AT-2), virus was incubated for 4 hours at 37°C with different concentrations formaldehyde (Sigma) (Darnell et al., 2004; Rossio et al., 1998), glutaraldehyde (Sigma) (Darnell et al., 2004) or AT-2 (Aldrich) (Chertova et al., 2003; Rossio et al., 1998). Formaldehyde, glutaraldehyde or AT-2 was afterwards removed by ultracentrifugation. For inactivation by changing the pH, the pH was adjusted with HCl and NaOH and virus was incubated for different times at a pH2 or at a pH12. After incubation, the pH was neutralized (Darnell et al., 2004). For temperature inactivation, virus was incubated for different times at 37°C (Darnell et al., 2004; Maheshwari et al., 2004). Inactivation of PRRSV with gamma irradiation was performed using an electron accelerator (Prof. L. Van Hoorebeke, Ghent University, Faculty of Science, Department of Subatomic and Radiation Physics). For PRRSV inactivation with ultraviolet (UV) radiation, virus was radiated with UV light from a UV cross-linker (UVP, Inc) (Darnell et al., 2004). Inactivation with binary ethyleneimine (BEI) was performed by incubating virus with 1 mM BEI (Aldrich) for different times at 37°C. The reaction was stopped with 0.1 mM sodium thiosulfate (Sigma) (Berhane et al., 2006; Mondal et al., 2005).

Analysis of virus inactivation

Virus titration was performed on 3 days cultivated Marc-145 cells following the standard procedure (Botner et al., 1999). After 5 days, occurrence of cytopathic effect (CPE) was investigated and the 50% tissue culture infective dose ($TCID_{50}$) was calculated.

To confirm that all virus was completely inactivated using selected inactivation procedures, either a complete dose (10^7 $TCID_{50}$/mL virus) or 10x more virus was inactivated and used for inoculation of Marc-145 cells, followed by two passages. The Marc-145 cells were investigated every week for CPE and cells were stained by immunoperoxidase monolayer assay (IPMA) (Labarque et al., 2000). Additionally, a bioassay was also done for selected inactivation methods by injecting 10^7 $TCID_{50}$/mL inactivated PRRSV in RPMI 1640 medium intramuscularly in a pig. Blood was taken every week and serum was checked for viremia by virus titration and PRRSV specific antibodies by IPMA up to 2 weeks post inoculation.

Effect of PRRSV inactivation on virus attachment, internalization, disassembly and infection by immunofluorescence staining

For the detection of PRRSV during the course of PRRSV infection, macrophages were inoculated with untreated or treated PRRSV (moi 2) for 1 hour at 4°C or 1, 5 and 10 hours at 37°C, fixed with methanol at -20°C and stained as described by Delputte et al. (Delputte et al., 2004). Briefly, the capsid protein was stained with a primary antibody P3/27 (Wieczorek-Krohmer et al., 1996) and a secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-mouse immunoglobulins (Molecular Probes). A virus stock with a ratio of 1/100 of infectious virus/non-infectious virus was used for inoculation. This is determined by application of diluted samples of virus suspension to glass slides, fixed with methanol and stained against nucleocapsid as described above. This showed that the virus stock with 10^6 $TCID_{50}$/mL contained approximately 10^8 particles/mL. Confocal analysis was performed using a TCS SP2 laser scanning spectrum confocal system (Leica Microsystems GmbH, Heidelberg, Germany) to determine the amount of internalized PRRSV. The numbers of bound and internalized PRRSV were counted from the acquired images. The amount of internalized PRRSV of both treated and untreated virus in a macrophage were determined by counting the small fluorescently labeled dots and set relatively to the number of internalized untreated virus.

Inhibition of phagocytosis

Macrophages were pre-treated for 30 minutes with 0.1 µM wortmannin to block phagocytosis. Afterwards the macrophages were inoculated with untreated or treated virus for 1 hour at 37°C in the presence of 0.1 µM wortmannin. Then the cells were fixed with methanol at -20°C and stained as described by Delputte et al. (Delputte et al., 2004). Briefly, the capsid protein was stained with a primary antibody P3/27 (Wieczorek-Krohmer et al., 1996) and a secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-mouse immunoglobulins (Molecular Probes). Confocal analysis was performed to check for endocytosis.

Analysis of the viral proteins of inactivated PRRSV by SDS-PAGE and Western blotting

SDS-PAGE, Western Blotting and ECL of viral proteins were performed essentially as described by Delputte et al. (Delputte et al., 2007). The membranes were stained for 1 hour with a primary mouse monoclonal antibody against one of the proteins of LV, M (126.3) (Meulenberg et al., 1995), N (P3/27) (Wieczorek-Krohmer et al., 1996), GP5 (4BE12)

(Rodriguez et al., 2001), GP4 (122.29) (Meulenberg et al., 1997b), GP3 (P9A3-20) (Intervet) or pig polyclonal antibody.

Vaccination study

Experimental design

Piglets derived from a PRRS-negative farm were used and their PRRSV-negative status was confirmed by IPMA. Twenty four piglets were randomly divided into four groups. A first group (group A) was a mock-vaccinated control group and received 1 mL RPMI in 1 mL Incomplete Freund's Adjuvant (IFA, Sigma) at 6 and 10 weeks of age. The other three groups were vaccinated twice intramuscularly at 6 (primo vaccination) and 10 (booster vaccination) weeks of age. Group B was vaccinated with 2 mL of a commercial European-type inactivated PRRSV vaccine (Progressis®, Merial). Group C received 1 mL of UV-inactivated virus in 1 mL IFA and group D received 1 mL of BEI-inactivated virus in 1 mL IFA. Four weeks after the booster vaccination, all pigs were challenged intranasaly with10^6 $TCID_{50}$ LV. Blood was taken weekly after vaccination and at 3, 5, 7, 10, 14, 21, 28 and 35 days post challenge. Serum was collected and stored at -70°C.

Virus-specific antibody detection

PRRSV-specific serum antibody titers were determined by IPMA as described by Labarque et al. (2000). Briefly, fixed LV infected Marc-145 cells were stained with 10-fold dilution series of serum and secondary antibody peroxidase labeled goat anti-mouse immunoglobulin (Ig) (DakoA/S, Glostrup, Denmark). Afterwards 3-amino-9-ethylcarbazole (AEC) substrate (Sigma) was added and the cells were analyzed with a light microscope (Olympus Optical Co., Hamburg, Germany). The IPMA antibody titer was expressed as the reciprocal of the last dilution that resulted in a positive reaction.

Virus-neutralizing antibody detection

Virus-neutralizing (VN) antibody titers were detected by a seroneutralization (SN) test on Marc-145 cells (Labarque et al., 2000). Briefly, a 2-fold dilution series of serum was mixed with an equal volume of virus with a titer of $2x10^3$ $TCID_{50}$/mL and incubated for 1 hour at 37°C. The serum-virus mixture was then mixed with Marc-145 cells and seeded in a 96-well plate. The cells were analyzed for CPE at 7 days post inoculation. The VN antibody titer was defined as the reciprocal of the highest dilution that inhibited CPE in 50% of the inoculated wells.

Detection of viremia

Virus titers in serum were determined by virus titration on 24 hours cultivated alveolar macrophages, following the standard procedure (Labarque et al., 2000). After 3 days incubation at 37°C, the occurrence of CPE was investigated. Macrophages were fixed at 3 days post inoculation (dpi) and an immunoperoxidase staining with monoclonal antibody P3/27 against the nucleocapsid protein of PRRSV was performed to determine infected cells. The 50% tissue culture infective dose ($TCID_{50}$) was calculated.

Statistical analysis

Antibody and virus titers were analyzed by Kruskall-Wallis test, followed by Dunn's multiple comparisons test to determine differences between groups at different time points. $P<0.05$ 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).

3.1.4 RESULTS

Effect of different treatments on PRRSV infectivity

To test the potential of the different PRRSV inactivation treatments, purified virus (10^7 $TCID_{50}$/mL) was treated with formaldehyde, glutaraldehyde, AT-2, pH, 37°C, gamma irradiation, UV irradiation or BEI, using different concentrations, time periods and/or doses. Treated PRRSV was titrated to determine an inactivation curve (Fig. 3.1). There was no infectious virus detected when PRRSV was treated with the lowest concentration of formaldehyde (0.1 µg/mL) (Fig. 3.1A), the lowest gamma irradiation dose (0.25 kGy) (Fig. 3.1F), the lowest UV irradiation dose (100 mJ/cm^2) (Fig. 3.1G), or at the first time point investigated for inactivation with pH2 (1 hour) (Fig. 3.1D), pH12 (data not shown), and BEI (6 hours) (Fig. 3.1H). For glutaraldehyde, the amount of infectious virus decreased in function of the concentration and no infectious virus could be detected upon incubation with a concentration of 0.5 µg/mL glutaraldehyde or more (Fig. 3.1B). PRRSV was still infectious after 4 hours incubation at 37°C with the highest concentration of 2 mM AT-2 (Fig. 3.1C). For 37°C treatment, the amount of infectious virus decreased in a time dependent way and there was no infectious virus detected after 48 hours incubation at 37°C (Fig. 3.1E). For formaldehyde, glutaraldehyde and AT-2, the titer at time point 0 was lower than 10^7 $TCID_{50}$/mL, because of loss of some virus during ultracentrifugation. These results showed

that for all inactivation methods, except for AT-2, a minimal treatment that allowed complete virus inactivation could be determined.

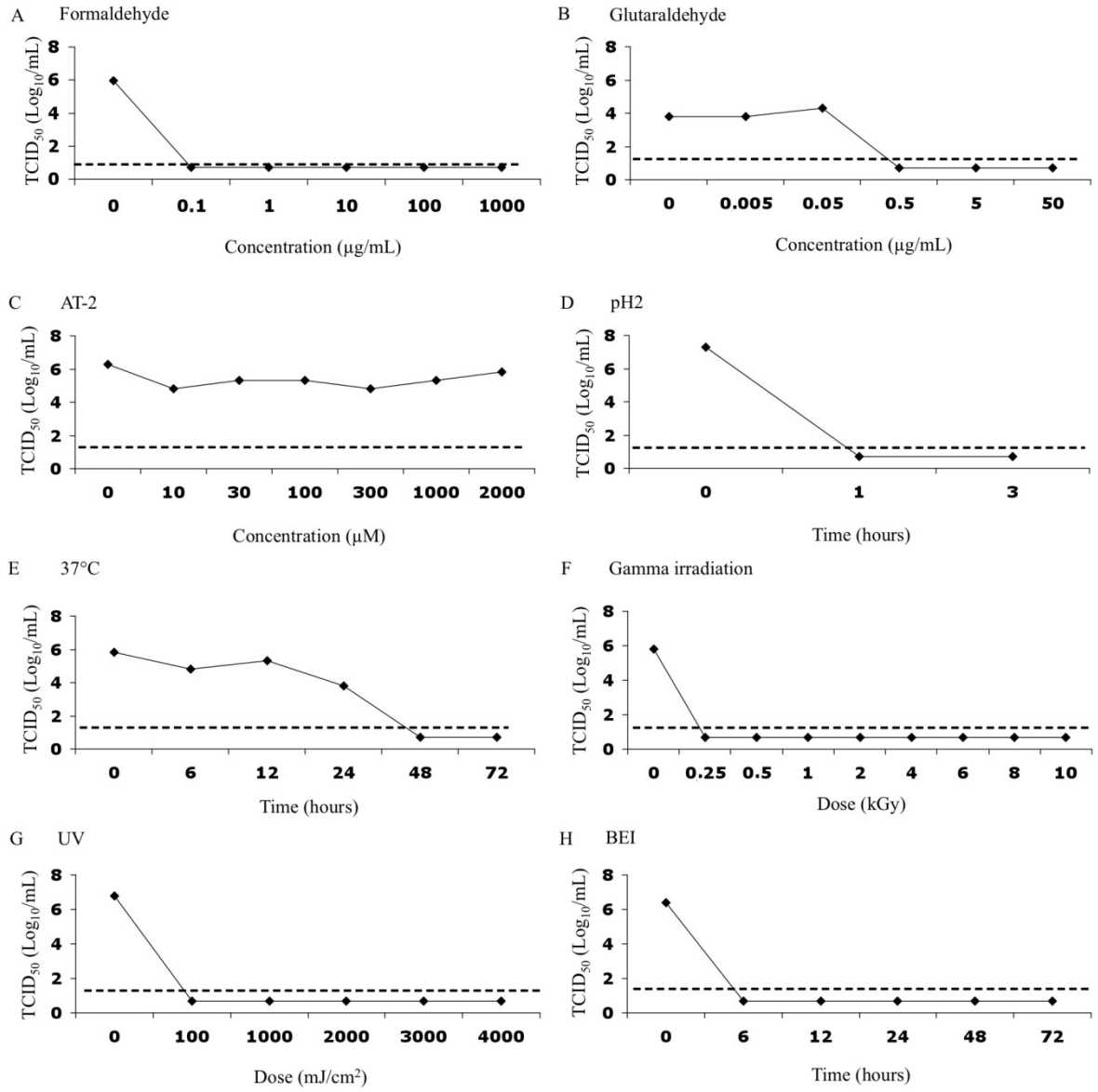

Figure 3.1. Effect of different inactivation methods on PRRSV infectivity. Untreated LV or inactivated LV was titrated on 3 days cultured Marc-145 cells to determine presence of infectious virus in the samples. The dotted line resembles the detection limit of the assay.

Effect of different inactivation procedures on virus internalization into macrophages

To investigate the preservation of the entry-associated domains of the treated PRRSV, an immunofluorescence staining was performed to determine whether internalization of inactivated PRRSV into macrophages was still possible. The results of these internalization experiments are shown in Figure 3.2 and Figure 3.3. PRRSV inactivated with 1 µg/mL formaldehyde (Fig. 3.2A and Fig. 3.3), 50 µg/mL glutaraldehyde (Fig. 3.2B and Fig. 3.3),

pH2 (Fig. 3.2D and Fig. 3.3) or pH12 (data not shown) for 1 hour was no longer able to internalize into macrophages. The internalization experiments with AT-2 inactivated PRRSV (Fig. 3.2C and Fig. 3.3) showed that PRRSV treated with all concentrations of AT-2 tested, could still internalize into macrophages, but this was to be expected since the virus was still infectious even at the highest concentration of AT-2. The internalization experiments for 37°C and BEI inactivation (Fig. 3.2E, H and Fig. 3.3) showed that PRRSV incubated at 37°C or treated with 1 mM BEI could still internalize into macrophages for all time points investigated. PRRSV treated with all doses of gamma irradiation or UV examined could still internalize into macrophages, but the internalization diminished in a dose dependent way for gamma irradiation inactivated PRRSV (Fig. 3.2F, G and Fig. 3.3).

Together, these data show that the entry-associated domains were not preserved when PRRSV is inactivated with formaldehyde, glutaraldehyde or pH changes, while they were preserved using AT-2, 37°C, gamma irradiation, UV or BEI.

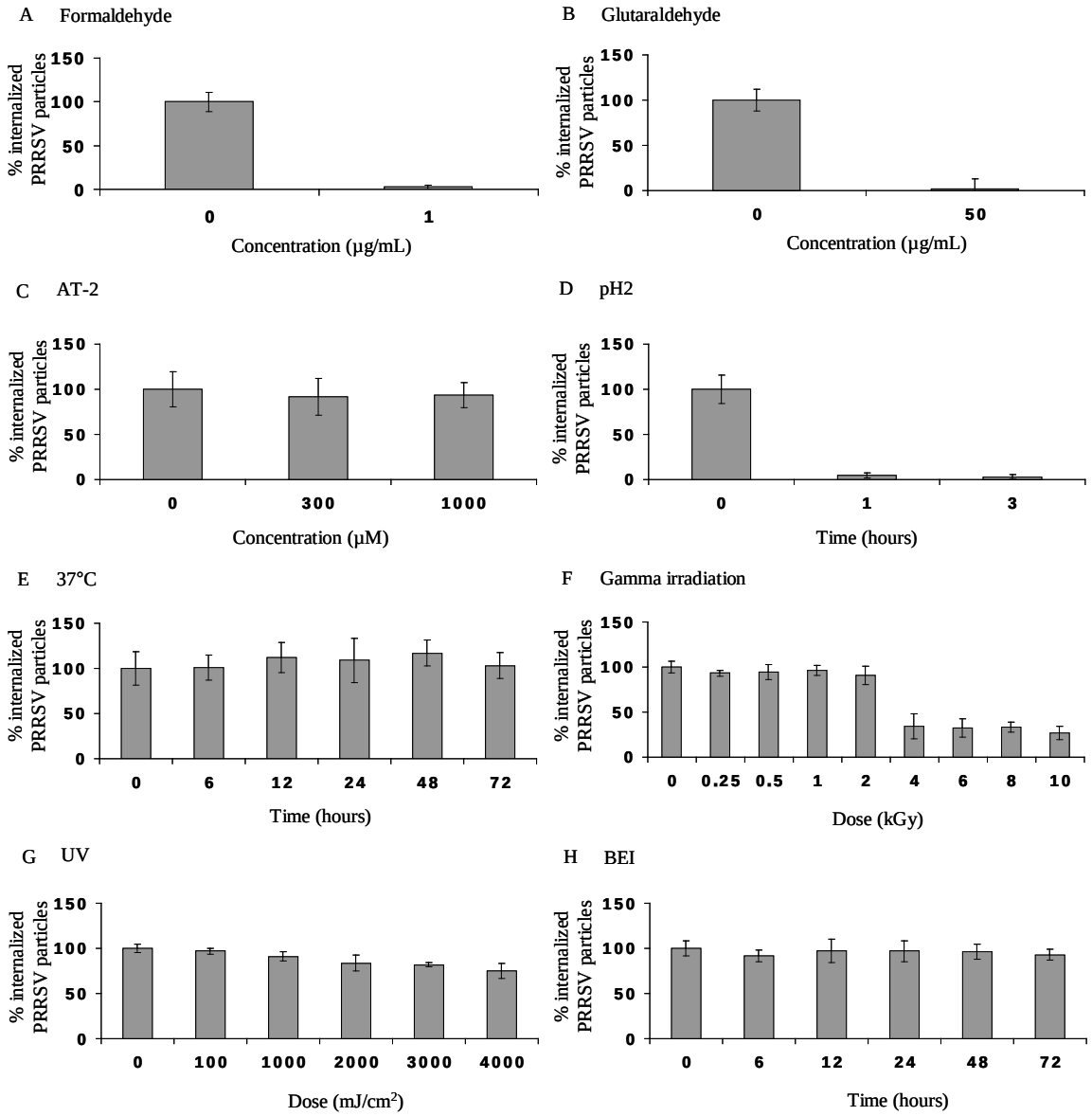

Figure 3.2. Effect of different inactivation methods on PRRSV internalization. Macrophages were incubated with untreated LV or inactivated LV for 1 hour. After 1 hpi, the virus was stained by immunofluorescence and internalization of PRRSV particles was determined by confocal microscopy. The amount of internalized PRRSV particles of both treated and untreated virus in a macrophage were counted by counting the small fluorescently labeled dots and set relatively to the number of internalized untreated virus.

Effect of the different inactivation methods on different stages in the virus replication cycle in macrophages

PRRSV inactivated with formaldehyde, glutaraldehyde and pH changes was not able to attache to and to internalize into macrophages, which is a crucial step in the viral replication cycle. Because internalization did not occur, the uncoating and virus replication did also not occur (Fig. 3.3).

PRRSV inactivated by 37°C, gamma irradiation, UV and BEI could still internalize into macrophages, thus it was able to perform this step of the viral replication cycle. After 5 hours,

the uncoating occurred, thus also the second step of the viral replication cycle could be performed. The inactivated virus was however not capable of replicating and this thus confirmed the completely inactivated status of the virus (Fig. 3.3).

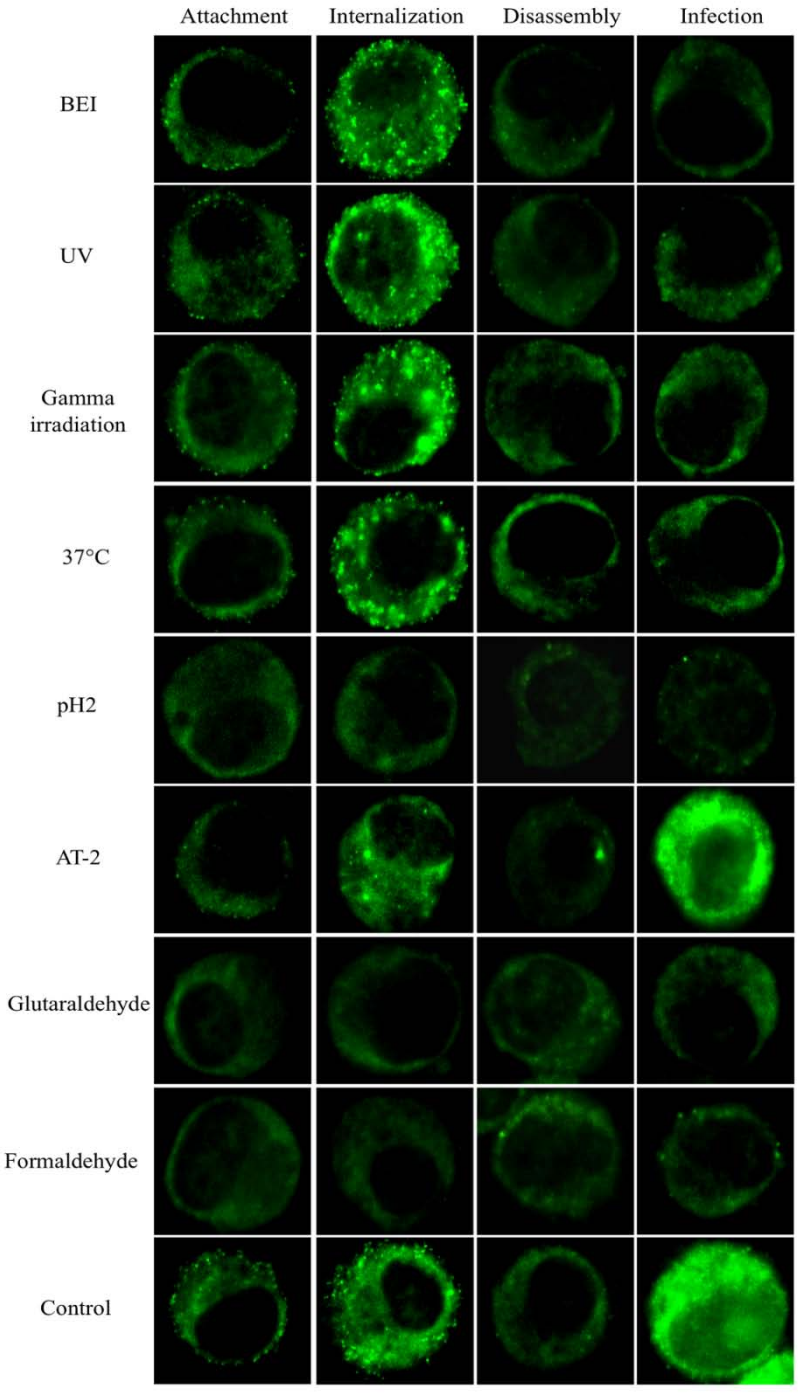

Figure 3.3. Effect of different inactivation methods on different stages of the viral replication cycle. Macrophages were incubated with inactivated LV or untreated LV for 1h at 4°C and 1, 5 or 10 h at 37°C. After fixing and immunofluorescence staining of the cells, attachment (1 hpi at 4°C), internalization (1 hpi at 37°C), disassembly (5 hpi at 37°C) and replication (10 hpi at 37°C) was measured by confocal microscopy.

Inhibition of phagocytosis to confirm receptor-mediated endocytosis after inactivation

To investigate if entry of the inactivated PRRSV in macrophages occurred via sialoadhesin and CD163 as described for infectious PRRSV (Van Gorp et al., 2008; Vanderheijden et al., 2003), and not simply taken up by phagocytosis, entry was also determined using a phagocytosis inhibitor wortmannin. After treatment of the macrophages with the phagocytosis inhibitor wortmannin, untreated PRRSV still attached to and internalized into macrophages. PRRSV inactivated with UV and BEI also attached to and internalized into macrophages, whereas formaldehyde and glutaraldehyde inactivated PRRSV could not attach and internalize.

Safety test to confirm complete inactivation

Gamma irradiation, UV and BEI seemed to be useful methods for killed PRRSV vaccine development, since the inactivated virus attaches and enters macrophages in a similar way as the virulent virus. Therefore, safety tests were done to confirm that the inactivated virus was completely inactivated and safe for use in pigs. First, 10^7 $TCID_{50}$/mL virus was inactivated and Marc-145 cells were inoculated for 2 passages with the inactivated virus. This test indicated that virus inactivated with 0.25 kGy gamma irradiation, 100 mJ/cm^2 UV or 6 hours incubation with BEI were completely inactivated (data not shown). To be sure the virus was inactivated, 1.5 kGy gamma irradiation, 1 000 mJ/cm^2 UV or 24 hours incubation with BEI were selected as a safe method to inactivate PRRSV. A second test was performed by inactivating a higher concentrated virus suspension under the same conditions (10^8 $TCID_{50}$/mL virus). The results demonstrated that 1 000 mJ/cm^2 UV or 24 hours incubation with BEI could still completely inactivate 10 times more virus (data not shown). Virus (10^8 $TCID_{50}$/mL virus) inactivated with gamma irradiation was not completely inactivated with a dose of 1.5 kGy and starting from a dose of 2 kGy, the preservation of entry-associated domains decreases. Therefore, inactivation with gamma irradiation is not useful for further vaccine development. Finally, to confirm complete inactivation, a bioassay was performed. Therefore, 10^7 $TCID_{50}$/mL virus was inactivated with 1 000 mJ/cm^2 UV or 24 hours incubation with BEI and intramuscularly injected in pigs. Viremia was not detected up to 2 weeks after injection, while virus-specific antibodies were induced, which suggests that the virus was completely inactivated and safe to use in pigs (data not shown).

Western blot analysis of the viral proteins of inactivated PRRSV

The effect of different inactivation methods on viral proteins was further investigated by analyzing the protein pattern of the virus before and after inactivation by Western blotting (Fig. 3.4, Table 3.1). Under reducing as well as under non-reducing circumstances, all the viral proteins were present after inactivation with formaldehyde, AT-2 and gamma irradiation, but to a lesser extent as untreated PRRSV. For glutaraldehyde, under reducing as well as under non-reducing conditions not all the viral proteins could be detected after inactivation. The protein pattern of the virus before and after inactivation by UV irradiation was not identical, which suggests that UV irradiation resulted in the degradation of the viral proteins. For pH changes, 37°C and BEI all the viral proteins were present after inactivation. Complexes like N-dimer and M-GP5 however were more degraded in comparison to the untreated virus when treated with pH changes.

The formaldehyde, glutaraldehyde, pH, AT-2, gamma irradiation and UV inactivated PRRSV did not have the same protein pattern as the untreated virus. These results indicated that with these inactivation procedures inactivated PRRSV did not resemble infectious PRRSV. The 37°C and BEI inactivated PRRSV showed the same protein pattern as the untreated virus. These results suggest that PRRSV inactivated at 37°C or by BEI resembles infectious PRRSV.

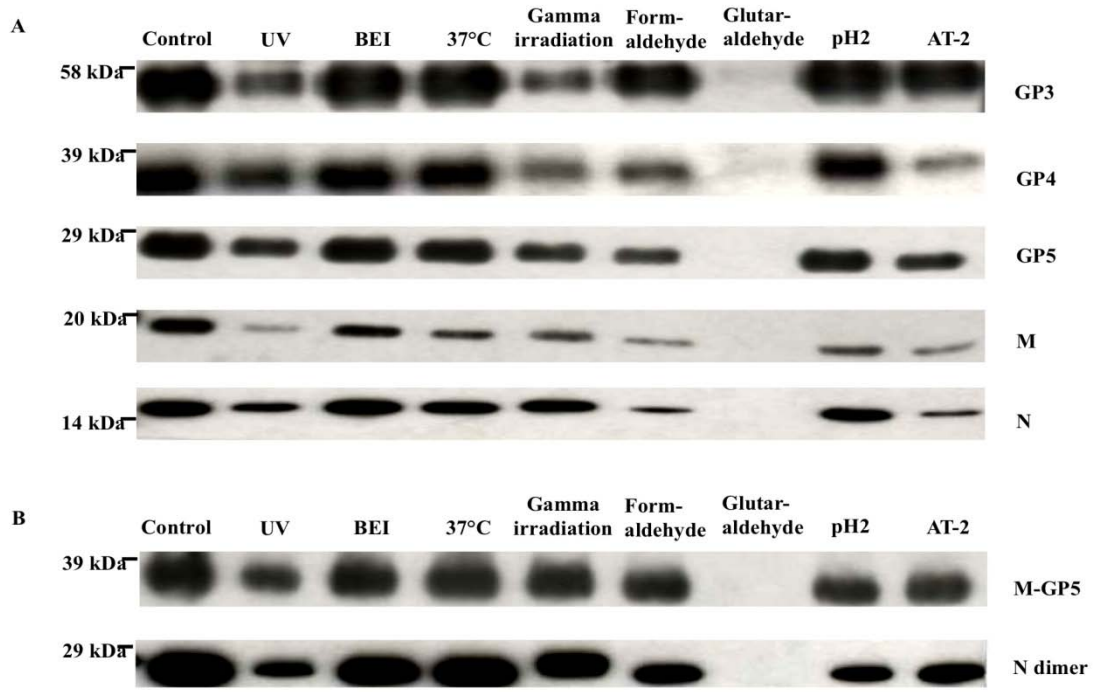

Figure 3.4. Effect of different inactivation methods on PRRSV proteins. Western blot analysis of untreated LV or inactivated LV in reducing (A) and non-reducing (B) conditions.

Table 3.1. Density of Western blots

	Control	UV	BEI	37°C	Gamma irradiation	Form-aldehyde	Glutar-aldehyde	AT-2	pH2
N	100	48	103	85	83	30	6	26	82
M	100	14	65	46	48	23	6	23	40
GP5	100	56	95	87	56	42	1	50	75
GP4	100	73	93	91	50	50	13	28	83
GP3	100	47	94	95	39	83	5	83	94
N dimer	100	45	83	92	66	43	0	43	31
M-GP5	100	58	87	92	80	73	0	63	67

Vaccination study

Virus-specific antibodies

In the mock-vaccinated control group (group A) no virus-specific antibodies were detected by IPMA in any of the pigs before challenge (Fig. 3.5). Of the pigs vaccinated with the commercial killed virus vaccine (group B), one pig transiently showed a positive IPMA antibody titer after vaccination and another had seroconverted at the day of challenge, while the other pigs remained seronegative up till the time of challenge. In contrast, all pigs vaccinated with UV-inactivated (group C) or BEI-inactivated (group D) virus showed positive antibody titers at three weeks after the primo vaccination. After the booster vaccination antibody titers rose to values as high as normally seen after PRRSV infection in naïve pigs (Labarque et al., 2000). After challenge, all pigs had seroconverted at day 10. Antibody titers in group B were slightly higher than in group A, however differences were not significant. In group C as well as group D, antibody titers were significantly higher compared to group A starting from one week after booster vaccination up till one week post challenge.

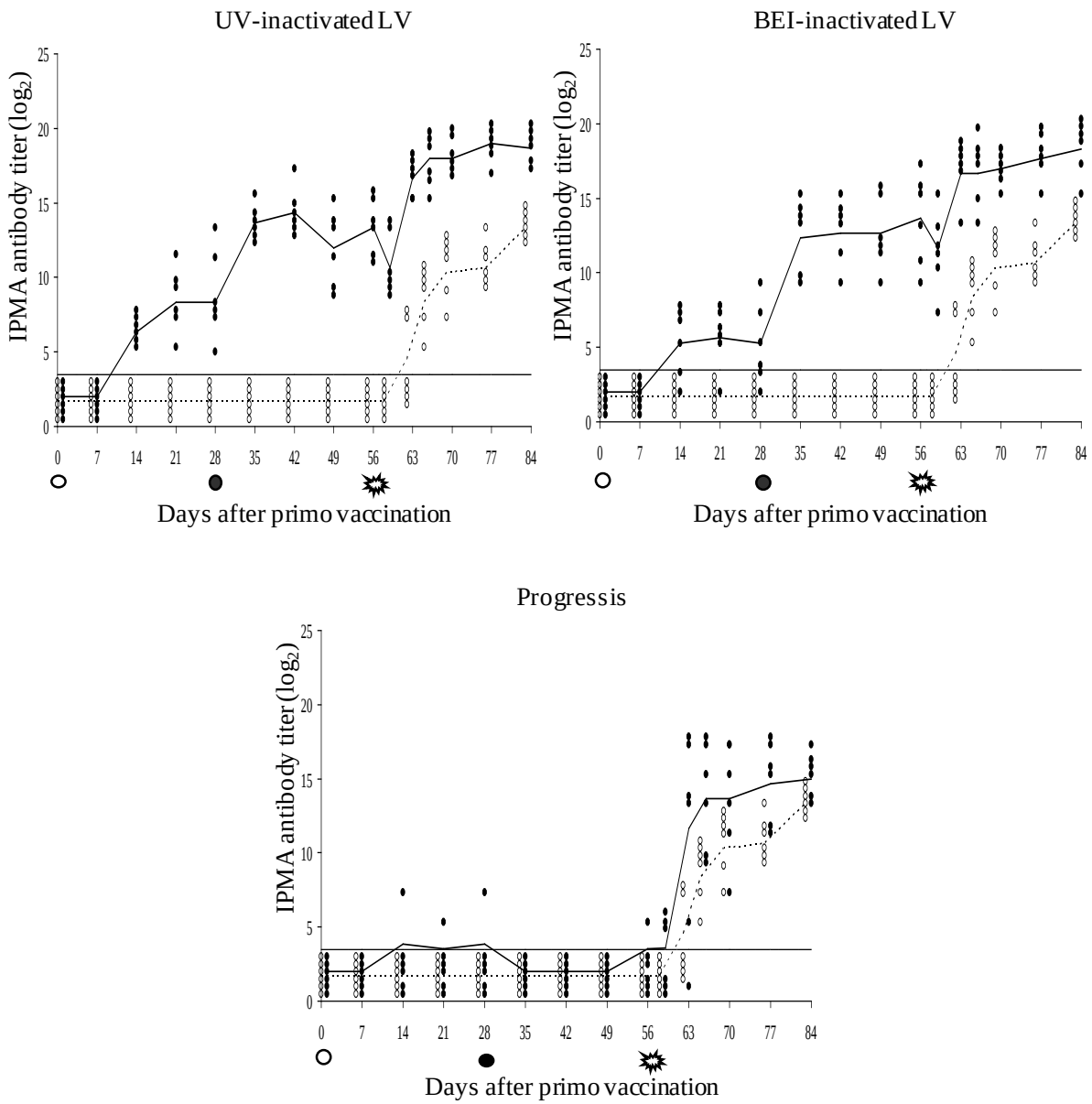

Figure 3.5. PRRSV-specific IPMA antibody titers (log2) after vaccination and after challenge for group A (adjuvant control, represented in each graph), B (commercial inactivated vaccine), C (UV-inactivated LV) and D (BEI-inactivated LV).
Virus-specific antibodies were determined with an IPMA test. The IPMA titers of the animals from the control group are represented by open symbols, those from the vaccinated group by filled symbols. The dotted line gives the mean IPMA titer for the control group. The full line gives the mean for the vaccinated group. The dashed line gives the detection limit. ○ = primo vaccination, ● = booster vaccination, ✸ = challenge

Virus-neutralizing antibodies

None of the pigs showed VN antibodies before challenge, except for one pig of group D that showed an SN antibody titer of 1.0 $\log_2$ two weeks after booster vaccination and at the day of challenge. One pig of group A showed a transient SN antibody titer at day 10 post challenge, however for all other pigs from group A, VN antibodies only appeared between three and five weeks post challenge. One pig even remained negative during the entire experiment (Fig. 3.6).

Only four pigs of group B showed VN antibodies after challenge, two starting from 10 days, one at four weeks and another at five weeks post challenge, and VN antibody titers did not differ between group A and B at any time point. In contrast, all pigs of group C and D showed VN antibodies after challenge. In group C, five animals seroconverted for VN antibodies within the first two weeks after challenge, and the sixth one after five weeks. VN antibody titers were significantly higher in group C compared to group A at two weeks post challenge, reaching a mean SN titer of 2.9 $\log_2$. In group D, one pig already had VN antibodies before challenge and starting from one week post challenge all pigs of this group showed a positive SN antibody titer, except for one animal that became positive one week later. VN antibody titers were significantly higher in group D compared to group A at 10, 14 and 21 days post challenge, reaching mean values of 2.6, 2.5 and 2.0 $\log_2$ respectively.

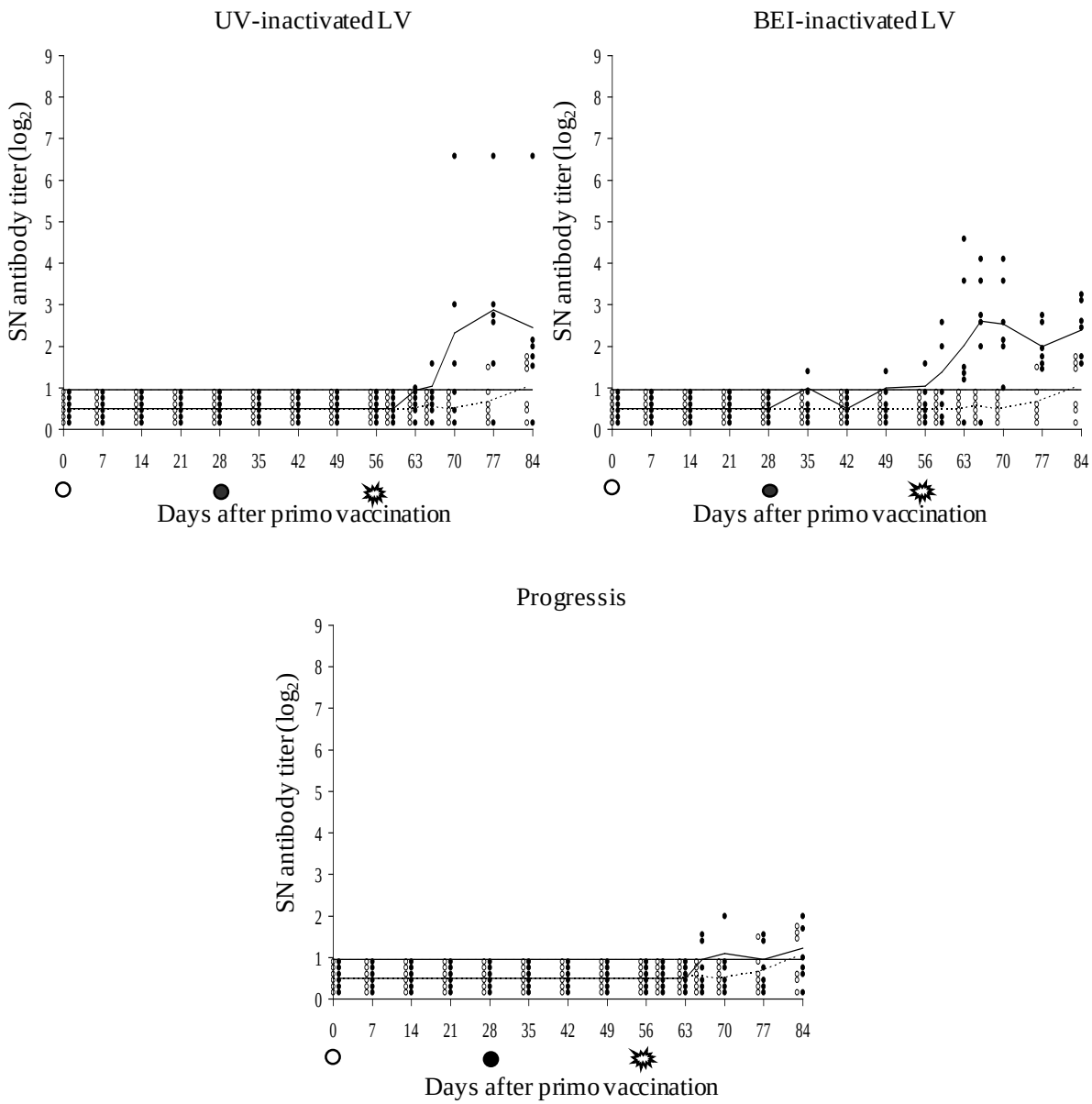

Figure 3.6. PRRSV-neutralizing antibody titers (log_2) after vaccination and after challenge for group A (adjuvant control, represented in each graph), B (commercial inactivated vaccine), C (UV inactivated virus) and D (BEI inactivated virus).
Virus-neutralizing antibodies were determined with an SN test. The SN titers of the animals from the control group are represented by open symbols, those from the vaccinated group by filled symbols. The dotted line gives the mean SN titer for the control group. The full line gives the mean for the vaccinated group. The dashed line gives the detection limit. ○ = primo vaccination, ● = booster vaccination, ✵ = challenge

Viremia

Virus was not detected in serum at one week after each immunization and at the day of challenge, confirming that all vaccines were properly inactivated. Figure 3.7 represents the virus titers (log_{10} $TCID_{50}$/mL) post challenge. In group A, a maximum mean virus titer of 3.5 log_{10} $TCID_{50}$/mL was reached at day 5 post challenge and a second peak of 3.1 log_{10} $TCID_{50}$/mL was observed at day 10. This was followed by a decline until all pigs were virus-

negative at four weeks post challenge. Mean virus titers in group B were slightly lower compared to those in group A, being 3.1 $\log_{10}$ $TCID_{50}$/mL at 5 days and 2.0 $\log_{10}$ $TCID_{50}$/mL at 10 days post infection, but no significant differences could be detected at any time point between group A and group B. The mean virus titer for group C was 2.7 $\log_{10}$ $TCID_{50}$/mL at 5 days and 2.5 $\log_{10}$ $TCID_{50}$/mL at 10 days post challenge, however titers were not significantly different from those in group A at any time point. Finally, the mean virus titer for group D was 2.6 $\log_{10}$ $TCID_{50}$/mL at 5 days post challenge and 1.4 $\log_{10}$ $TCID_{50}$/mL at day 10, and at the latter time point virus titers were significantly reduced, compared to group A.

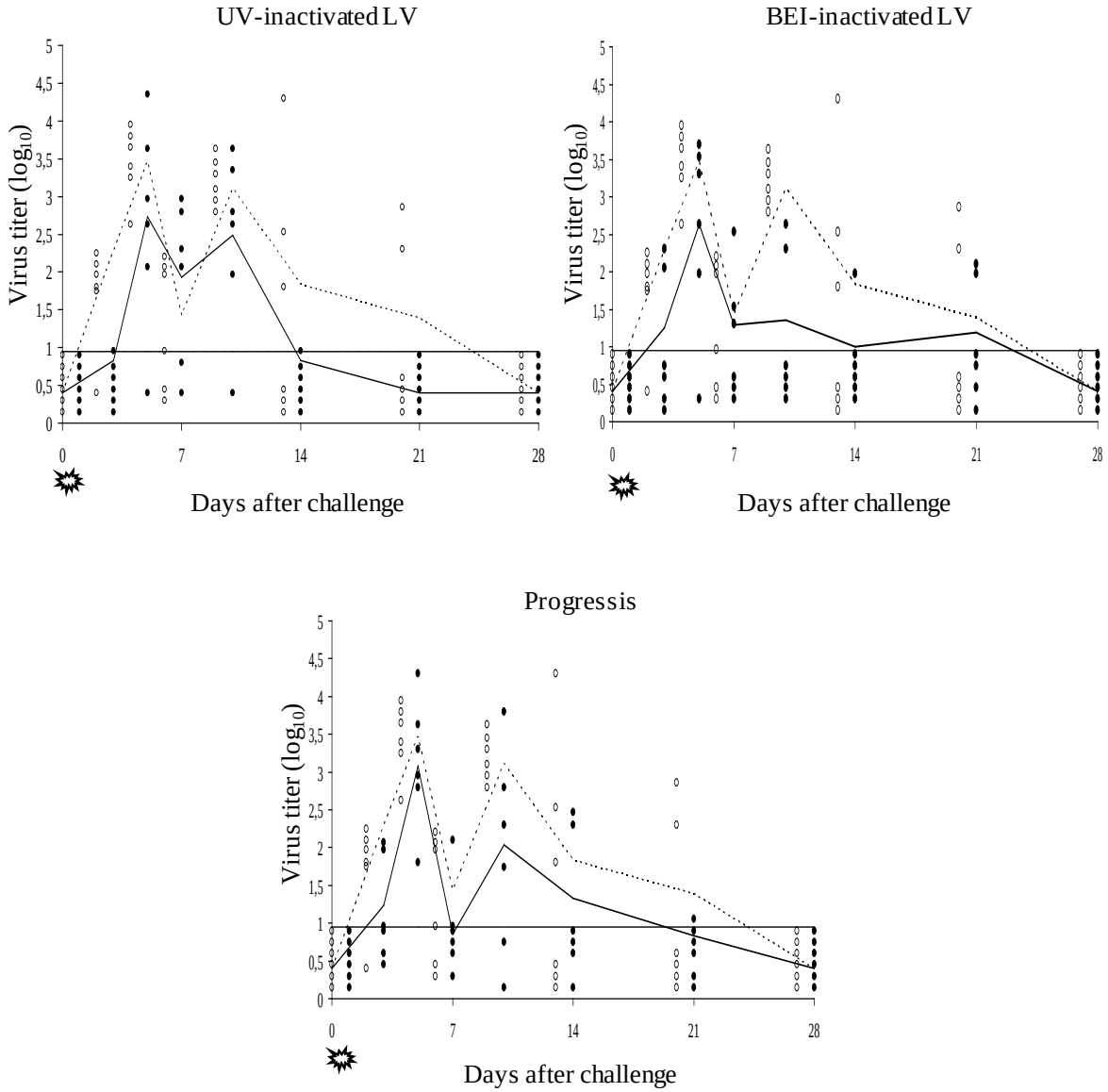

Figure 3.7. Serum virus titers after challenge for group A (adjuvant control, represented in each graph), B (commercial inactivated vaccine), C (UV inactivated virus) and D (BEI inactivated virus).
Virus titers were determined by virus titration. The virus titers of the animals from the control group are represented by open symbols, those from the vaccinated group by filled symbols. The dotted line gives the mean virus titer for the control group. The full line gives the mean for the vaccinated group. The dashed line gives the detection limit. ✵ = challenge

3.1.5 DISCUSSION

Virus inactivation procedures with the aim to develop inactivated vaccines should have two major goals: complete inactivation of infectious virus (safety), while conserving epitopes of the inactivated virus that are important for the induction of protective immunity (antigen quality). This study aimed to test different inactivation procedures for PRRSV to evaluate the effect on both reduction of infectivity and conservation of viral entry-associated domains, the latter being a measure for the quality of the antigen used.

Currently, there are no data available in literature on inactivation procedures of PRRSV. Therefore, inactivation methods and conditions used in this study are based on studies where inactivation of other viruses was evaluated. Evaluation of the capacity of different inactivation procedures to completely inactivate PRRSV showed similarities and differences with that of other viruses. PRRSV could not be inactivated with AT-2, not even after treatment with 2 mM for 4 hours at 37°C, while HIV type 1 is already inactivated with 100 µM AT-2 after 1 hour at 37°C (Rossio et al., 1998). AT-2 modifies free thiol groups of internal viral proteins like the nucleocapsid of HIV-1, more specifically zinc-finger motifs important for HIV-1 infection, leaving disulfide bridges of glycoproteins in the virus envelope unaffected (Chertova et al., 2003; Williams et al., 2002), but for PRRSV the formation of homodimers of nucleocapsid proteins via disulfide bridges is important for virus infection (Wootton and Yoo, 2003). Since PRRSV seems not to be sensitive to AT-2, this product cannot be used to develop an inactivated PRRSV vaccine. For PRRSV inactivation with formaldehyde and glutaraldehyde, respectively 0.1 µg/mL and 0.5 µg/mL for 4 hours at 37°C was sufficient. In comparison, HIV-1 can be inactivated with 2 µg/mL formaldehyde after 24 hours at 37°C (Rossio et al., 1998) and SARS-CoV with 90 µg/mL formaldehyde or 20 µg/mL glutaraldehyde after 24 hours at 37°C (Darnell et al., 2004). For PRRSV inactivation by changing the pH, incubation of the virus at a pH2 or pH12 for 1 hour at 37°C was effective. Also SARS-CoV could be inactivated after incubation of 1 hour at 37°C at a pH2 or pH12 (Darnell et al., 2004). Incubation of PRRSV for 48 hours at 37°C was efficient for its inactivation, while SARS-CoV was inactivated after incubation of 20 minutes at 56°C (Darnell et al., 2004) and adenovirus type 5 after 10 minutes at 50°C (Maheshwari et al., 2004). For this study, inactivation of PRRSV at higher temperatures was not considered, since PRRSV proteins, similar to what is observed with other viruses (Schlegel et al., 2001), will most likely be denaturated, thereby destroying important epitopes. PRRSV inactivation with gamma irradiation or UV could be achieved with an irradiation dose of respectively 0.25 kGy or 100

mJ/cm^2. SARS-CoV could not be inactivated with gamma irradiation, even not after an irradiation dose of 15 000 rad. For inactivation with UV, an irradiation dose of 3614 mJ/cm^2 was needed (Darnell et al., 2004). BEI inactivation of PRRSV could be achieved with 1 mM BEI after 6 hours incubation at 37°C. Berhane et al. (Berhane et al., 2006) inactivated Nipah virus with 3 mM BEI for 24 hours at room temperature and Mondal et al. (Mondal et al., 2005) used 1.6 mM BEI for 24 hours at 37°C to inactivate rabies virus. In conclusion, for all tested inactivation procedures, except for AT-2, conditions that allowed PRRSV inactivation could be determined.

Besides inactivation, conservation of viral entry-associated domains is equally important for the development of an inactivated vaccine. An *in vitro* assay to check the conservation of viral domains that are important for the induction of a protective immunity is preferred to avoid time consuming vaccination studies and to allow precise fine tuning of inactivation methods. For influenza virus and HIV for example, the major neutralizing epitopes are known and an *in vitro* assay to analyze the conservation of these domains after inactivation can be performed by measuring hemagglutination for influenza virus (Di Trani et al., 2003) or by ELISA for HIV (Grovit-Ferbas et al., 2000). However, for PRRSV there is currently no *in vitro* assay to evaluate this due to a limited knowledge on the PRRSV neutralizing epitopes. In our lab, it was shown that neutralizing antibodies were preventing infection by disturbing entry of the virus into macrophages (Delputte et al., 2004). Therefore, it was hypothesized that viral domains important for viral entry (entry-associated domains) are also important for the induction of viral neutralizing antibodies. In this study, different inactivation methods for PRRSV were analyzed for their effect on the viral domains important for entry into macrophages, which are most likely also important for the induction of neutralizing antibodies. Our experiments with formaldehyde, glutaraldehyde and pH inactivated PRRSV suggest that the viral entry-associated domains are modified, since the virus can no longer attach to and internalize into macrophages. Similarly, Western blotting showed that the viral proteins were not or to a lesser extent present, which is probably due to cross-linking of proteins by formaldehyde and glutaraldehyde or denaturation of viral proteins by pH changes. Formaldehyde and glutaraldehyde are known to have a similar effect on proteins, as they are able to induce protein cross-linking (Alderson, 1964; Cheung and Nimni, 1982; Fraenkel-Conrat, 1954), while pH changes affect proteins by denaturation (Weismiller et al., 1990). Cross-linking or denaturation of viral domains that are involved in attachment and internalization of PRRSV might interfere with the subsequent presentation of viral domains to cells of the adaptive immune system. As a result, inactivation with formaldehyde,

glutaraldehyde and pH changes will probably give a poor preservation of viral immunogenicity (Cranage et al., 1995; Rossio et al., 1998). In conclusion, formaldehyde, glutaraldehyde and pH changes are not effective methods to inactivate PRRSV with preservation of the entry-associated domains and for future vaccine development.

Because gamma irradiation mainly has an effect at the genomic level, one could assume that this would be an effective method to inactivate PRRSV. However, the range between complete inactivation and preservation of the entry-associated domains was too small to use this method in a safe way for vaccine development. PRRSV inactivated with a dose of 1.5 kGy was still infectious, while PRRSV inactivated with a dose of 2 kGy was no longer infectious, but could not efficiently internalize into macrophages. At this dose viral entry-associated domains are most likely destroyed due to the formation of free radicals which damage proteins (Grieb et al., 2002). This viral protein degradation was also seen on Western blot. In conclusion, gamma irradiation is not suitable as a method to inactivate PRRSV.

High temperature inactivation has been documented to inactivate viruses by denaturation (Lelie et al., 1987; Schlegel et al., 2001). As with protein cross-linking, denaturation of viral proteins may also destroy the entry-associated domains of PRRSV. Thermal inactivation of viruses by RNA degradation via breakage of the phosphodiester bond is also described (Fleming, 1971). Virus inactivation at ‘low’ temperature (below 41°C) is considered to be caused by degradation of the nucleic acid, whereas virus inactivation at ‘high’ temperature is related to protein denaturation (Dimmock, 1967; Fleming, 1971; Laude, 1981). A modest increase of temperature (37°C) did not prevent PRRSV to attach to and internalize into macrophages, while preventing viral replication. Incubating PRRSV at 37°C would thus be an interesting option to generate a killed PRRSV vaccine. Clearly, inactivation at 37°C does not result in modification of viral proteins. This is confirmed by Western blotting, since no difference was observed in the banding pattern between not inactivated control virus and inactivated virus.

Of all methods tested, the most promising methods to inactivate PRRSV for KV vaccine development are UV and BEI, because they mainly have an effect on genomic level, preserving entry-associated viral domains. This was shown by internalization of the inactivated virus into macrophages. However, for UV the destruction of capsid proteins has been reported (Miller and Plagemann, 1974). Western blot analysis in this study indeed shows degradation of viral proteins upon UV inactivation.

By investigating internalization into macrophages, the possibility exists that the inactivated virus is taken up by phagocytosis instead of using the PRRSV entry receptor sialoadhesin.

However, PRRSV inactivated by UV and BEI did also attach to and internalized into macrophages after treatment with a phagocytosis inhibitor. These results confirm that inactivated PRRSV could still interact with PRRSV receptors and that thus internalization of BEI or UV inactivated PRRSV into macrophages did not occur via phagocytosis.

In summary, the results of this study shows that inactivation methods that have a direct effect on viral proteins, like formaldehyde, glutaraldehyde and changing the pH are not good candidates for viral inactivation, as they do not conserve viral entry-associated domains. On the other hand, UV, BEI and gamma irradiation, which mainly have an effect on the genome, could be interesting methods to inactivate PRRSV for vaccine development, as inactivated virus is still able to internalize into macrophages, but no longer replicates. However, UV and gamma irradiation may give problems with photoproducts or free radicals formed during irradiation, which in turn could indirectly degrade viral proteins. This study also revealed that exposure of PRRSV to a modest increased temperature was an efficient method of viral inactivation.

To investigate if the methods that seem the most suitable according to the *in vitro* screening assay described in this study are indeed good for preserving the immunogenicity of PRRSV inactivated vaccines, new experimental inactivated PRRSV vaccines based on UV and BEI inactivation were developed and tested in animals (Vanhee et al., 2009). The efficacy of the experimental UV- and BEI-inactivated vaccines were compared with a commercial, European-type killed PRRSV vaccine. Vaccination with both UV- and BEI-inactivated virus strongly induced virus-specific antibodies in all animals and resulted in an earlier and elevated VN antibody response after infection. The strong priming of the VN antibody response by vaccination with UV- or BEI-inactivated virus suggests the presence of intact neutralizing antigens on the vaccine virus. In contrast, vaccination with the commercial vaccine did not induce virus-specific antibodies, and only resulted in a slightly elevated antibody response after challenge. The latter results are similar to a study showing that vaccination with this commercial vaccine only resulted in an anamnestic humoral immune response after challenge (Zuckermann et al., 2007). Vaccination with the commercial inactivated PRRSV vaccine did not influence the VN antibody response after challenge. This is in contrast with the study showing an anamnestic VN antibody response in pigs that were vaccinated with this vaccine (Scortti et al., 2007; Zuckermann et al., 2007). Virus was cleared from the blood in control animals between two and four weeks post infection. Animals that were vaccinated with the commercial inactivated PRRSV vaccine showed a slight reduction in viremia compared to control animals. In contrast, there was an early decline in virus titers observed in animals that

were vaccinated with UV- or BEI-inactivated virus. All experimental vaccines tested were able to reduce viremia, starting from one week post infection. Moreover, viral clearance was systematically observed at earlier time points in vaccinated animals, compared to control animals.

The efficacy of two experimental inactivated PRRSV vaccines, developed on basis of a new quality test for neutralizing antigens showed that vaccination of naïve pigs with UV- or BEI-inactivated PRRSV strongly primed the VN antibody response and resulted in a reduction of viremia. This new quality test can contribute to the development of safe and effective PRRSV vaccines that offer the opportunity to include emerging field strains.

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Chapter 4

EFFICACY OF INACTIVATED PRRS VIRUS PRODUCED ON SIALOADHESIN AND CD163 TRANSFECTED CELL LINES

4.1 CONSTRUCTION AND CHARACTERIZATION OF CHO$^{SN\text{-}CD163}$ AND PK15$^{SN\text{-}CD163}$ CELL LINES, SUSCEPTIBLE TO PRRSV

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4.1.1 SUMMARY

Porcine reproductive and respiratory syndrome virus (PRRSV) causes major economic losses in the pig industry worldwide. *In vivo*, the virus infects a subpopulation of tissue macrophages. *In vitro*, PRRSV only replicates in primary pig macrophages and African green monkey kidney derived cells, such as Marc-145. The latter is currently used for vaccine production. However, since virus entry in Marc-145 cells is different compared to entry in primary macrophages, specific epitopes associated with virus entry could potentially alter upon growth on Marc-145 cells. To avoid this, we constructed CHO and PK15 cell lines recombinantly expressing the PRRSV receptors involved in virus entry into macrophages, sialoadhesin (Sn) and CD163 ($CHO^{Sn\text{-}CD163}$ and $PK15^{Sn\text{-}CD163}$) and evaluated their potential for production of PRRSV. Detailed analysis of PRRSV infection revealed that LV and VR-2332 virus particles could attach to and internalize into the $CHO^{Sn\text{-}CD163}$ and $PK15^{Sn\text{-}CD163}$ cells. Initially, this occurred less efficiently for macrophage grown virus than for Marc-145 grown virus. Upon internalization, disassembly of the virus particles was observed. The two cell lines could be infected with PRRSV strains LV and VR-2332. However, it was observed that Marc-145 grown virus infected the cells more efficiently than macrophage grown virus. If the cells were treated with neuraminidase to remove cis-acting sialic acids that hinder the interaction of the virus with Sn, the amount of infected cells with macrophage grown virus increased. Comparison of both cell lines showed that the $PK15^{Sn\text{-}CD163}$ cell line gave in general better results than the $CHO^{Sn\text{-}CD163}$ cell line. Only 2 out of 5 tested PRRSV strains replicated well in $CHO^{Sn\text{-}CD163}$ cells. Furthermore, the virus titer of all 5 PRRSV strains produced after passaging in $PK15^{Sn\text{-}CD163}$ cells was similar to the virus titer of those strains produced in Marc-145 cells. Analysis of the sequence of the structural proteins of original virus and virus grown for 5 passages on $PK15^{Sn\text{-}CD163}$ cells showed either no amino acid (aa) changes (VR-2332 and 07V063), one aa (LV), two aa (08V194) or three aa (08V204) changes. None of these changes are situated in known neutralizing epitopes. Together, the results show that this cell line can be used to produce vaccine virus and for PRRSV virus isolation.

4.1.2 INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the family *Arteriviridae*, order *Nidovirales* (Cavanagh, 1997; Snijder and Meulenberg, 1998) causing major economic losses in the pig industry worldwide (Neumann et al., 2005). PRRSV infection may result in reproductive failure in sows and is involved in the porcine respiratory disease complex (PRDC) (Christianson et al., 1993; Christianson et al., 1992; Lager and Mengeling, 1995; Mengeling et al., 1994; Rossow et al., 1994; Terpstra et al., 1991).

In vivo, the virus infects a subpopulation of tissue macrophages (Duan et al., 1997a; Duan et al., 1997b; Labarque et al., 2000; Molitor et al., 1997). *In vitro*, efficient PRRSV replication is only observed in primary pig macrophages (e.g. alveolar macrophages) (Wensvoort et al., 1991), differentiated monocytes (Delputte et al., 2007a) or African green monkey kidney derived cells, such as Marc-145 (Kim et al., 1993; Wensvoort et al., 1991). 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 macrophages via heparan sulphate (Delputte et al., 2002), then the virus is internalized via sialoadhesin (Sn) (Vanderheijden et al., 2003). CD163 is also involved in infection of macrophages, probably at the stage of virus disassembly (Van Gorp et al., 2008). PRRSV infection of Marc-145 cells occurs via binding to a heparin-like molecule as a first step (Jusa et al., 1997). The nucleocapsid of PRRSV is described to bind to the intermediate filament vimentin, which is suggested to mediate transport of the virus to the cytosol (Kim et al., 2006). CD151 may be involved in fusion of the viral envelope and the endosome, but the precise mechanism is still unknown (Shanmukhappa et al., 2007). CD163 is also essential for PRRSV infection of Marc-145 cells, but its role in this process is still unclear (Calvert et al., 2007).

Currently, PRRS vaccine virus is produced in Marc-145 cells. However, since virus entry in Marc-145 cells is different compared to entry in primary macrophages (Delputte et al., 2004) and because adaptation is needed for growth on Marc-145 cells (Collins et al., 1992), it is possible that specific epitopes associated with virus neutralization are lost or modified. Although virus production in primary macrophages would be ideal to avoid adaptation, these cells cannot be used because of batch variation, risk of contamination with other pathogens present in the macrophages isolated from pigs and high production costs. Previous results in our lab showed that non-permissive cells transiently transfected with Sn only sustained internalization, but not infection (Vanderheijden et al., 2003). Non-permissive cells

transiently transfected with CD163 allow a low level of infection depending on the cell type used (Calvert et al., 2007). Co-expression of both Sn and CD163 is the most efficient for PRRSV infection in different cell lines evaluated (Van Gorp et al., 2008).

To avoid the problems associated with PRRS vaccine virus production in other cell types, the aim of this study was to construct cell lines that recombinantly express Sn, the receptor that mediates PRRSV attachment to and internalization into macrophages (Delputte et al., 2005; Vanderheijden et al., 2003) and CD163, which is most probably involved in virus disassembly in macrophages (Van Gorp et al., 2008). Both Sn and CD163 are needed to make a PRRSV susceptible cell line for virus production that mimics the natural entry pathway in macrophages.

4.1.3 MATERIALS AND METHODS

Cells, viruses and plasmids

CHO-K1 cells were cultivated in F12 medium and PK15 cells in Dulbecco Modified Eagle Medium (D-MEM). Both media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate and a mixture of antibiotics. The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. Macrophages cultivated in medium containing RPMI-1640, 10% FBS, 2 mM L-glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate and a mixture of antibiotics were used for titration. A pcDNA3.1D/V5-HisTOPO plasmid containing Sn cDNA and geneticine resistance gene (Vanderheijden et al., 2003) and a pBUD plasmid with CD163 cDNA and zeocin resistance gene were used for transfection. To construct the pBUD plasmid containing CD163, CD163 from a pcDNA3.1D/V5-HisTOPO plasmid containing CD163 (Van Gorp et al., 2008), was cloned into a pBUD plasmid via restriction site HindIII and XbaI. The European prototype PRRSV strain Lelystad virus (LV), grown on Marc-145 cells and macrophages (Wensvoort et al., 1991), the American prototype PRRSV strain VR-2332 grown on Marc-145 cells (Collins et al., 1992), and three recent Belgian isolates, belonging to the European type, grown on macrophages (07V063, 08V204 and 08V194) were used for inoculation.

Transfection and selection

CHO-K1 and PK15 cells were transfected with FuGENE 6 (Roche) or Lipofectamine Plus (Invitrogen, Merelbeke, Belgium) respectively, according to the manufacturer's instructions. CHO-K1 and PK15 cells were first transfected with a plasmid containing the Sn cDNA and geneticine resistance gene. The cells were single cell cloned and selected for Sn expressing CHO and PK15 cells with geneticine (200µg/mL, GIBCO). Afterwards, the obtained CHO^{Sn} and PK15^{Sn} cells were transfected with a plasmid containing the CD163 cDNA and zeocin resistance gene and single cell cloned. For the selection of CHO^{Sn} and PK15^{Sn} cells expressing CD163, zeocin (200µg/mL, Invitrogen, Merelbeke, Belgium) was used.

Screening of cells expressing Sn and CD163 by immunofluorescence staining

Transfected CHO-K1 and PK15 cells were fixed with methanol and stained with primary monoclonal antibodies (mAb) against Sn (mAb 41D3) (Duan et al., 1998; Vanderheijden et al., 2003) and CD163 (mAb 2A10, AbD Serotec) (Bullido et al., 1997; Sanchez et al., 1999). As a secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-mouse immunoglobulins (Invitrogen, Molecular Probes, Merelbeke, Belgium) were used. Screening of cells expressing Sn and/or CD163 was performed with a fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany).

Analysis of PRRSV infection of CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells by immunoperoxidase staining

CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells were seeded in 96-well plates and inoculated with virus. At different time points post inoculation (pi), the cells were fixed with methanol and an immunoperoxidase staining was performed (Delputte et al., 2002). Briefly, viral antigen positive cells were stained with primary mAb anti-nucleocapsid P3/27 (Wieczorek-Krohmer et al., 1996) and secondary antibody peroxidase labeled goat anti-mouse immunoglobulin (Ig) (DakoA/S, Glostrup, Denmark). Afterwards, 3-amino-9-ethylcarbazole (AEC) substrate (Sigma, Bornem, Belgium) was added. The amount of infected cells was counted with a light microscope (Olympus Optical Co., Hamburg, Germany).

Attachment, internalization, disassembly and infection of CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells with PRRSV analyzed by immunofluorescence staining

To determine attachment, internalization, disassembly and infection of CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells, the cells were seeded at 200 000 cells/mL and after 2 days of cultivation

they were inoculated with virus. The cells were fixed with methanol after 1 hour incubation at 4°C to investigate attachment, since virus is not able to internalize at 4°C. The cells were fixed after 1 hour incubation at 37°C to determine the internalized particles. After 5 hours incubation at 37°C, the cells were fixed to analyze disassembly (disappearance of staining). To analyze infection, the cells were fixed after 24 hours incubation at 37°C. The virus was stained with a primary mAb anti-nucleocapsid P3/27 (Wieczorek-Krohmer et al., 1996) and a secondary FITC-conjugated goat polyclonal anti-mouse immunoglobulins (Invitrogen, Molecular Probes, Merelbeke, Belgium). Virus particles were counted on images acquired with a TCS SP2 laser scanning spectrum confocal system (Leica Microsystems GmbH, Heidelberg, Germany).

Virus production after passaging in CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells

CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells were seeded at 200 000 cells/mL in Tissue Culture (TC) flasks. After 2 days of cultivation, the cells were initially inoculated with 10^5 $TCID_{50}$ of each virus strain. After 3 and 5 days post inoculation (dpi), the supernatant was collected and centrifuged for 10 minutes at 30xg at 4°C. The supernatant was stored at -70°C and titrated. Virus titration was performed on 24 hours cultivated alveolar macrophages following the standard procedure (Labarque et al., 2000). After 3 days of incubation at 37°C, the occurrence of cytopathic effect (CPE) was investigated. Macrophages were fixed at 3 dpi and an immunoperoxidase staining was performed to identify infected cells. The 50% tissue culture infective dose ($TCID_{50}$) was calculated.

Virus sequencing after passaging virus in PK15$^{Sn\text{-}CD163}$ cells

RNA was extracted from PRRSV passaged 4 times on PK15$^{Sn\text{-}CD163}$ cells using an 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'-cggcccaittccatccigag-3') and ORF4-REV (5'-cattcagctcgcataicgtcaag-3'), ORF5-FW2 (5'-tgcticatttcitgacacc-3') and ORF5-REV1 (5'-accttaagigcitatatc-3'), ORF6FW (5'-taccaactttcttctggac-3') and ORF6REV (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 Biosystems, Foster City, USA) and PRRSV primers. Cycle sequencing reaction products were purified by ethanol precipitation and separated on an ABI Genetic 310 (Applied Biosystems, Foster City, USA).

The sequences were analyzed and compiled by BlastN and BlastP (www.ncbi.nlm.nih.gov), and Sixframe, ClustalW, Align (workbench.sdsc.edu). The Genbank accession numbers are for 07V063 [Genbank:GU737264], for 08V204 [Genbank:GU737266] and for 08V194 [Genbank:GU737265].

4.1.4 RESULTS

Construction of CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cell line

CHO-K1 and PK15 cells were transfected with Sn and CD163 and selected for cells expressing both receptors as shown in Figure 4.1A. The presence of Sn and CD163 was confirmed by immunofluorescence staining (Fig. 4.1B). 16 CHO and 4 PK15 clones co-expressing Sn and CD163 (CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$) were selected. 10 CHO and 4 PK15 clones were obtained, in which 100% of the cells retained stable expression of Sn and CD163 for at least 15 passages. The other 6 CHO clones lost either Sn or CD163 expression after a few passages. After a preliminary screening for PRRSV susceptibility, 3 CHO clones (IC5, ID9 and IF3) and 2 PK15 clones (IXH7 and IXA3) were retained for further analysis.

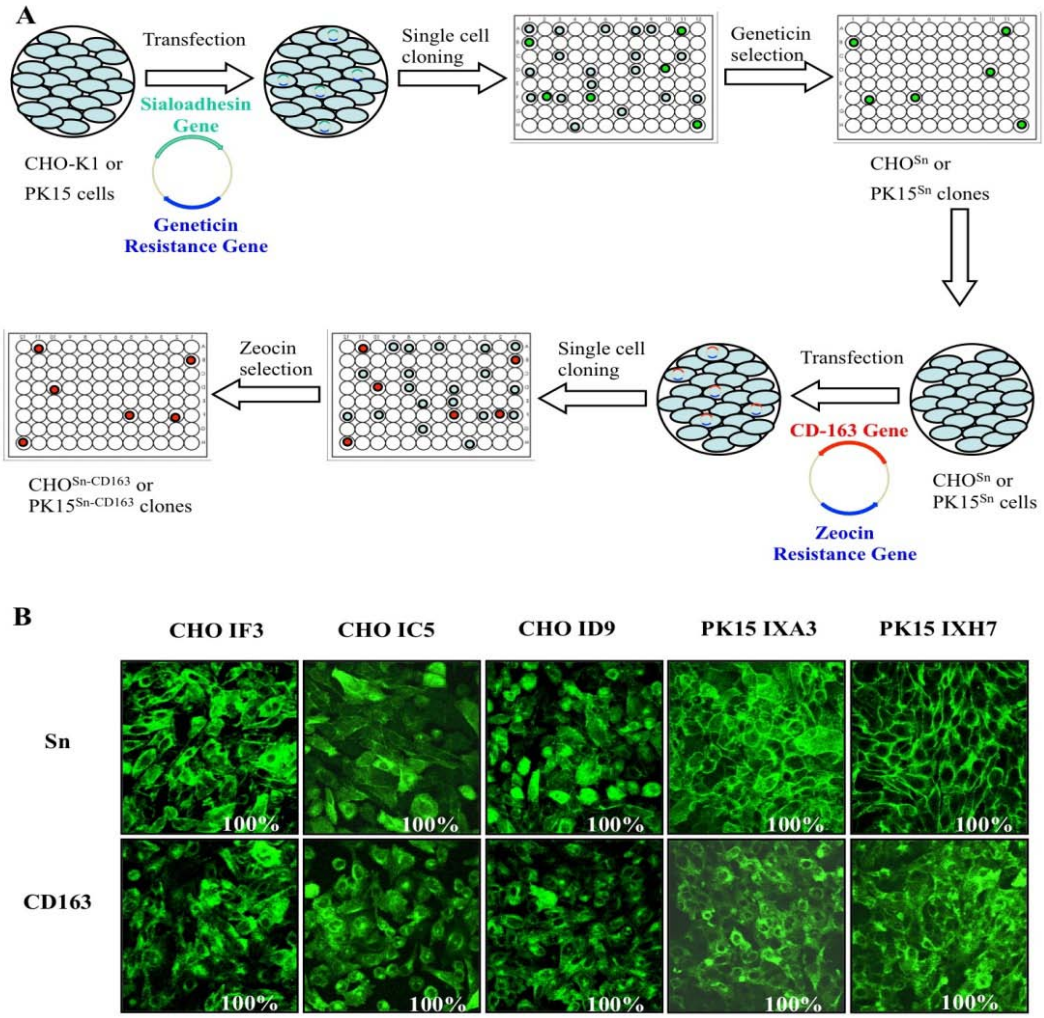

Figure 4.1. A) Schematic representation of the construction of CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cell lines. To construct a cell line co-expressing Sn and CD163, CHO-K1 or PK15 cells were transfected with a plasmid containing the Sn cDNA and a geneticine resistance gene. The cells were single cell cloned and clones were screened for Sn expressing cells. After selection for geneticine resistance, the obtained CHO^{Sn} or PK15^{Sn} cells were transfected with a plasmid containing the CD163 cDNA and a zeocin resistance gene, which allowed selection of cells expressing both Sn and CD163. B) Immunofluorescence staining of the obtained CHO$^{Sn\text{-}CD163}$ or PK15$^{Sn\text{-}CD163}$ cells for Sn and CD163. Some CHO$^{Sn\text{-}CD163}$ clones (IF3, IC5 and ID9) and PK15$^{Sn\text{-}CD163}$ clones (IXA3 and IXH7) are represented with their Sn and CD163 expression.

Effect of cell density and cultivation time of CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells on the susceptibility to PRRSV infection

To determine the effect of cell density and cultivation time of the cells on susceptibility to PRRSV infection, 3 CHO$^{Sn\text{-}CD163}$ cell clones (IC5, ID9 and IF3) and 2 PK15$^{Sn\text{-}CD163}$ cell clones (IXH7 and IXA3) were seeded at different cell densities (100 000, 200 000 or 300 000 cells/mL) and inoculated with 50 µL containing 10^4 $TCID_{50}$ Marc-145 grown LV, Marc-145 grown VR-2332 or macrophage grown LV at different days post seeding (1, 2 or 3 days post seeding). After 2 dpi, the cells were fixed and stained.

For Marc-145 grown virus infection of CHO$^{Sn\text{-}CD163}$ cells, little difference was observed between different cell densities and days post seeding, although a density of 200 000 cells/mL and inoculation at 2 days post seeding seemed a little more efficient. The VR-2332 strain infected the CHO$^{Sn\text{-}CD163}$ cells more efficiently than the LV strain. For PK15$^{Sn\text{-}CD163}$ cells, the infection rate for Marc-145 grown LV as well as VR-2332 was approximately 80%, independently of densities and cultivation time. CHO$^{Sn\text{-}CD163}$ clones IC5, ID9 and IF3 were equally sensitive to virus infection. There was no difference in sensitivity for infection between PK15$^{Sn\text{-}CD163}$ clones IXH7 and IXA3. The PK15$^{Sn\text{-}CD163}$ cells could be infected more efficiently than the CHO$^{Sn\text{-}CD163}$ cells (Fig. 4.2).

Both CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells showed a low infection rate with macrophage grown virus. The cell density had no influence on infection. There were no differences between clone IC5, ID9 and IF3 for CHO$^{Sn\text{-}CD163}$ and between IXH7 and IXA3 for PK15$^{Sn\text{-}CD163}$ cells (Fig. 4.2). Overall, Marc-145 grown virus strains could infect the cells more efficiently than macrophage grown virus strains tested.

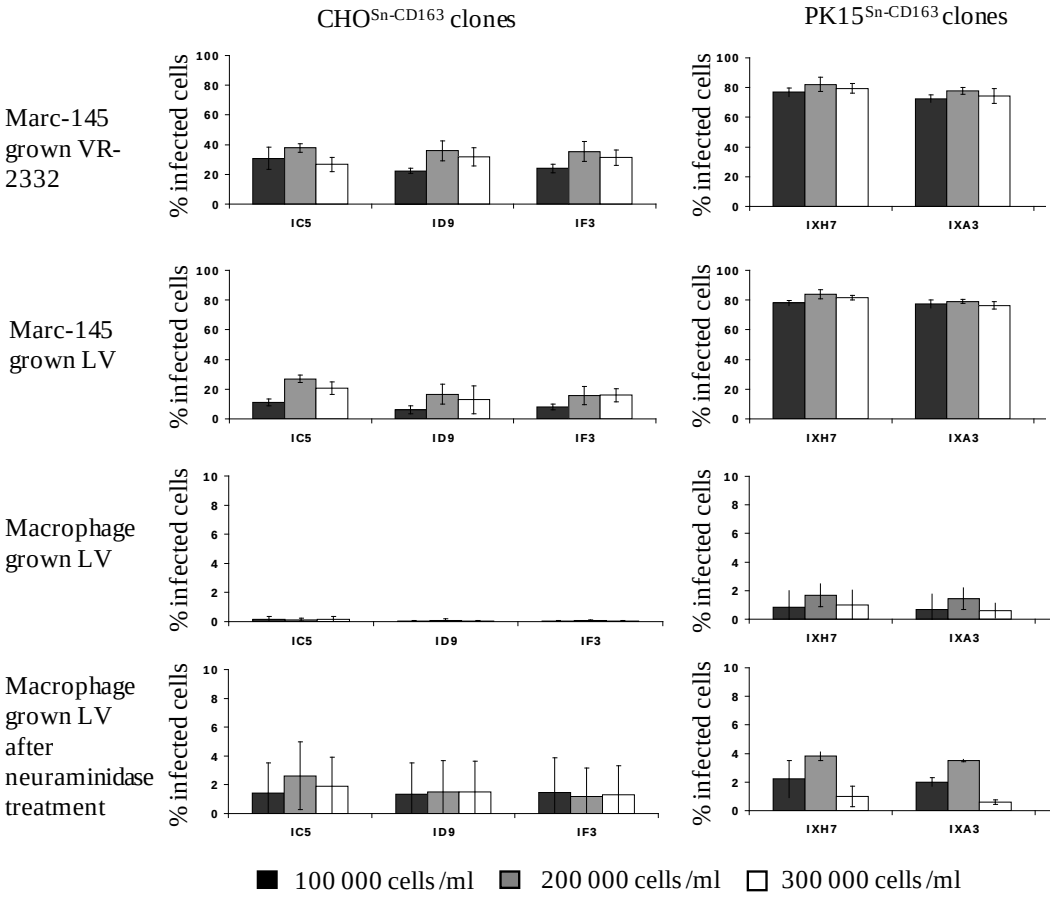

Figure 4.2. Effect of cell density on susceptibility of CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells to PRRSV infection. CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells were cultivated for 2 days before they were inoculated with Marc-145 grown LV, Marc-145 grown VR-2332 or macrophage grown LV. The black bars represent a cell density of 100 000 cells/mL, the grey bars 200 000 cells/mL and the white bars 300 000 cells/mL. The graphs show the percentage of infected cells. Values represent mean ± SD of three experiments.

Effect of neuraminidase treatment of CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells on the susceptibility to macrophage grown PRRSV infection

The previous section showed that macrophage grown PRRSV infection rate is very low. Previously, it was however shown that infection of macrophages can be enhanced after removal of sialic acid from the cells with neuraminidase, as observed in our lab (Delputte et al., 2007b). Desialylation of macrophages enhances sialoadhesin-mediated lectin activity (Barnes et al., 1999). Since the interaction between sialic acids on PRRSV and sialoadhesin is important for infection of cells (Delputte and Nauwynck, 2004), desialylation of the CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells can probably enhance the amount of infected cells by macrophage grown virus. Therefore, to increase the infection rate of macrophage grown virus infection, 3 CHO$^{Sn\text{-}CD163}$ cell clones (IC5, ID9 and IF3) and 2 PK15$^{Sn\text{-}CD163}$ cell clones (IXH7 and IXA3) were seeded at different densities (100 000, 200 000 or 300 000 cells/mL) and were infected at different days post seeding (1, 2 or 3 days post seeding) with 50 µL containing 10^4 $TCID_{50}$ macrophage grown LV, after treatment of the cells with neuraminidase to remove cis-acting sialic acids. After 2 dpi, the cells were fixed and stained. The results showed that treatment of the PK15$^{Sn\text{-}CD163}$ cells at 200 000 cells/mL with neuraminidase before inoculation enhanced infection of the cells with macrophage grown virus (Fig. 4.2).

Analysis of PRRSV attachment, internalization, disassembly and infection of CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells

To investigate if virus attachment, internalization, disassembly and infection occurs in the CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells, CHO$^{Sn\text{-}CD163}$ clone IC5 and PK15$^{Sn\text{-}CD163}$ clone IXH7 were inoculated with Marc-145 grown LV (moi 1), Marc-145 grown VR-2332 (moi 1) and macrophage grown LV (moi 1). The virus was stained by immunofluorescence at different stages of the viral replication cycle. First, the virus particles were clearly shown to attach to the cells. Then, virus particles were internalized into the cells, with macrophage grown virus being less efficient than Marc-145 grown virus. After internalization, the particles were uncoated to release the genome. Finally, infection occurred (Fig. 4.3). In general, Marc-145 grown virus infected the cells more efficiently than macrophage grown virus. Further, Marc-145 grown VR-2332 infected the PK15$^{Sn\text{-}CD163}$ cells more efficiently than Marc-145 grown LV, while this was equal for the CHO$^{Sn\text{-}CD163}$ cells. Infection of the cells with macrophage grown virus was very low. Treatment of the cells with neuraminidase before inoculation enhanced the infection rate of PK15$^{Sn\text{-}CD163}$ cells. The PK15$^{Sn\text{-}CD163}$ cells were infected most efficiently in all cases (Fig. 4.3).

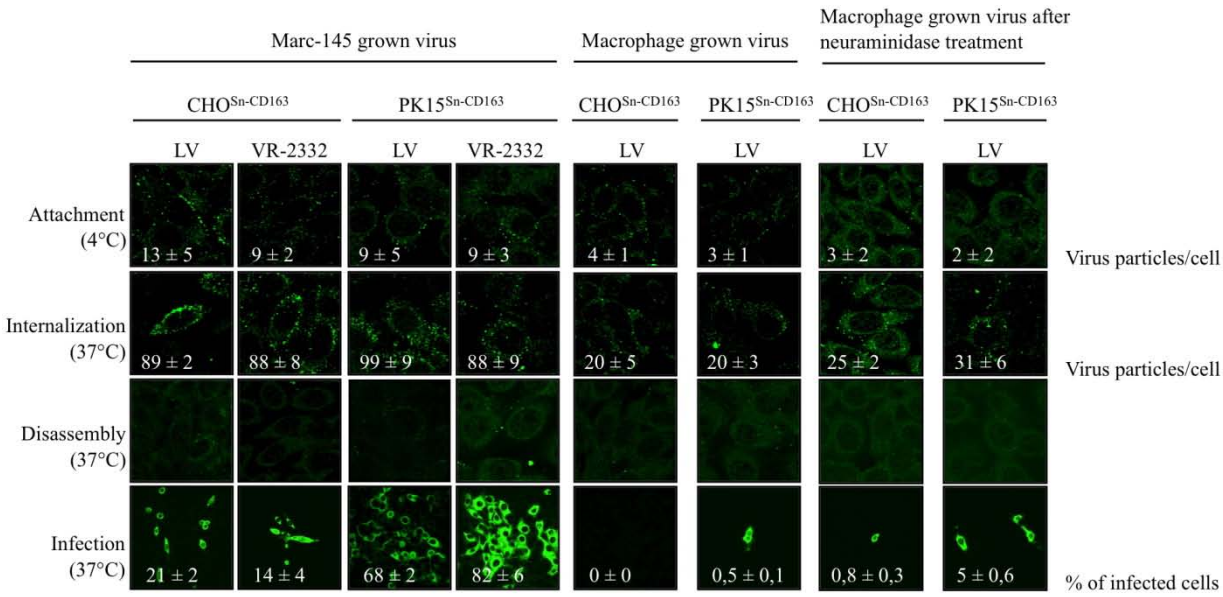

Figure 4.3. Attachment, internalization, disassembly and infection in CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells CHO$^{Sn-CD163}$ clone IC5 and PK15$^{Sn-CD163}$ clone IXH7 were inoculated with Marc-145 grown LV, Marc-145 grown VR-2332 or macrophage grown LV and different stages of the viral replication cycle were investigated by immunofluorescence staining of the virus.

PRRSV infection kinetics on CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells

To investigate the susceptibility of CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells to PRRSV infection, cells were seeded at 200 000 cells/mL and infected with Marc-145 grown LV, Marc-145 grown VR-2332 or macrophage grown LV at a moi of 0.2 at 2 days post seeding. For macrophage grown virus infection, a comparison was made between infection of cells treated with neuraminidase and untreated cells. The cells were fixed 1, 2, 3, 5 and 7 dpi and stained by immunoperoxidase. Figure 4.4 shows that Marc-145 grown VR-2332 could infect more CHO$^{Sn-CD163}$ cells than Marc-145 grown LV, while both strains infect PK15$^{Sn-CD163}$ cells for approximately 80%. Macrophage grown virus did infect a low number of cells for the two cell lines (Fig. 4.4). If the CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells were treated with neuraminidase, infection of the PK15$^{Sn-CD163}$ cells, but not the CHO$^{Sn-CD163}$ cells, with macrophage grown virus was increased (Fig. 4.4). At 3 and 5 dpi, the highest amount of virus infection was achieved, with up to 20% for Marc-145 grown LV and up to 40% for Marc-145 grown VR-2332 on CHO$^{Sn-CD163}$ cells and up to 80% for both Marc-145 grown strains on PK15$^{Sn-CD163}$ cells. The infection rate of cells infected with macrophage grown virus did not reach 5% in both cell lines. After treatment of the cells with neuraminidase, infection with macrophage grown virus increased on PK15$^{Sn-CD163}$ cells. In summary, Marc-145 grown LV and Marc-145 grown VR-2332 could infect the cell lines most efficiently. Macrophage grown virus gave

little infection, but if the cells were first treated with neuraminidase, infection was slightly better in the case of PK15$^{Sn\text{-}CD163}$ cells.

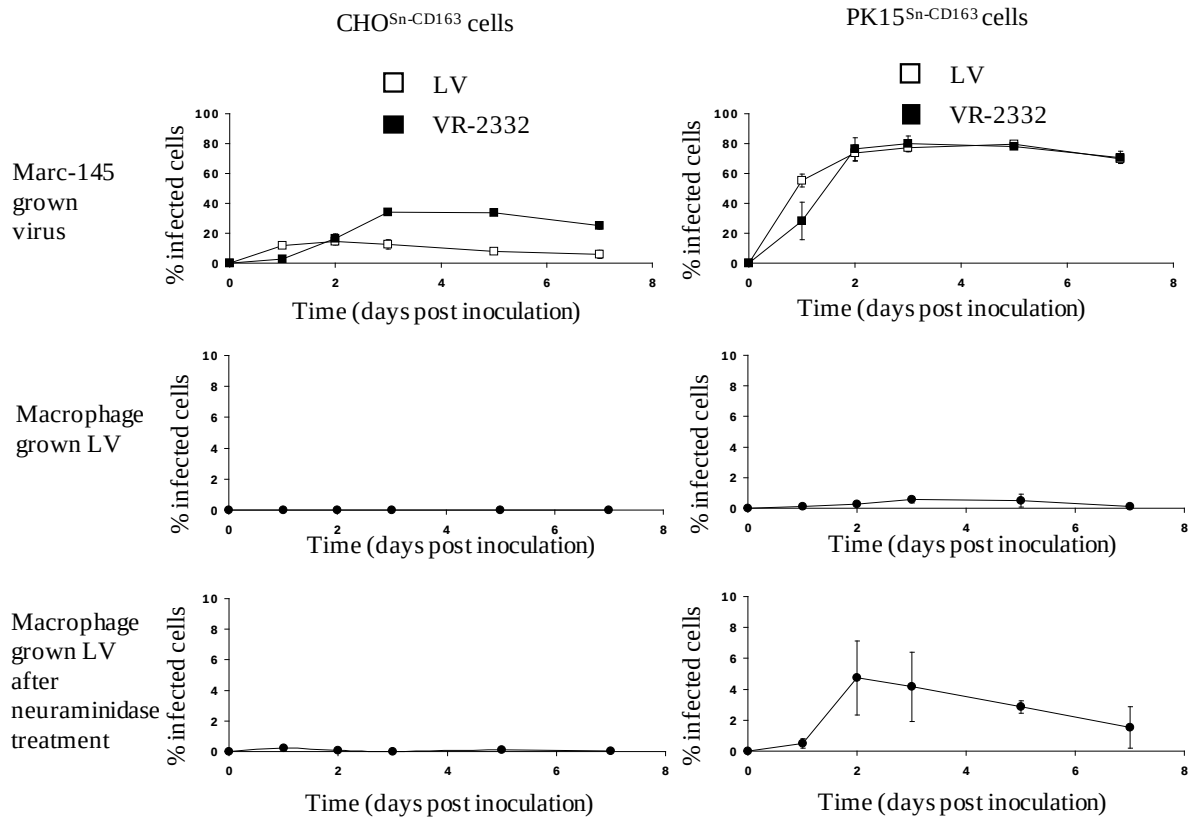

Figure 4.4. PRRSV infection kinetics in CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells. CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells were inoculated with Marc-145 grown LV (□), Marc-145 grown VR-2332 (■) or macrophage grown LV (●). After 1, 2, 3, 5 and 7 dpi the cells were fixed and an immunoperoxidase staining was performed. The amount of infected cells were counted and expressed in the graphs as the percentage of infected cells. Values represent mean ± SD of three experiments.

Virus production in CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells

To determine virus production in CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells, the cells were inoculated with Marc-145 grown VR-2332, macrophage grown LV, macrophage grown 07V063, macrophage grown 08V204 and macrophage grown 08V194. When the virus was passaged for several times, the virus titer increased, especially in PK15$^{Sn\text{-}CD163}$ cells, to reach a stable level starting from passage 3. A titer of $10^{6.0\pm0.3}$ and $10^{5.0\pm0.6}$ $TCID_{50}$/mL were obtained after 3 passages in CHO$^{Sn\text{-}CD163}$ cells for VR-2332 and 07V063 respectively. LV, 08V204 and 08V194 did not grow on CHO$^{Sn\text{-}CD163}$ cells. A titer of $10^{5.1\pm0.8}$, $10^{7.4\pm0.3}$, $10^{8.0\pm0.3}$, $10^{5.8\pm0.5}$ and $10^{6.5\pm0.3}$ $TCID_{50}$/mL was achieved after 3 passages in PK15$^{Sn\text{-}CD163}$ cells for LV, VR-2332, 07V063, 08V204 and 08V194 respectively. All virus titers of virus produced in both cell lines till passage 5 are represented in Figure 4.5. As a comparison, also virus yield obtained after 3 passages in Marc-145 cells was determined via titration on macrophages, revealing a titer of

$10^{6.6\pm0.3}$, $10^{6.8\pm0.6}$, $10^{7.1\pm0.6}$, $10^{5.5\pm0.6}$ and $10^{7.1\pm0.3}$ $TCID_{50}$/mL for LV, VR-2332, 07V063, 08V204 and 08V194 respectively. Similar virus titers are thus produced in PK15$^{Sn\text{-}CD163}$ and Marc-145 cells.

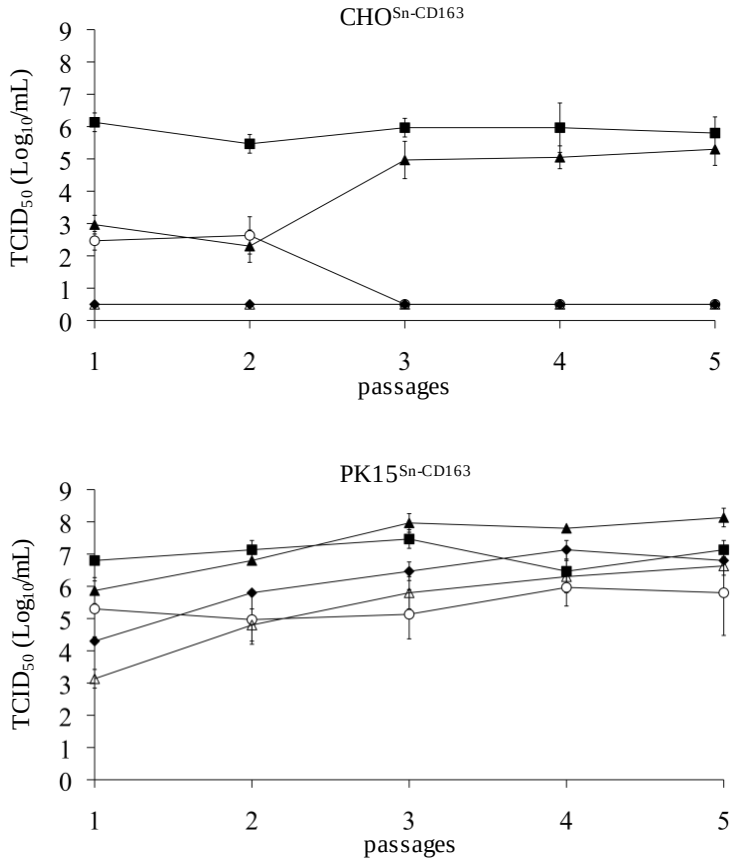

Figure 4.5. Virus production on CHO$^{Sn\text{-}CD163}$ and PK15$^{Sn\text{-}CD163}$ cells. CHO$^{Sn\text{-}CD163}$ clone IC5 and PK15$^{Sn\text{-}CD163}$ clone IXH7 were inoculated with Marc-145 grown VR-2332 (■), macrophage grown LV (○), macrophage grown 07V063 (▲), macrophage grown 08V204 (△) or macrophage grown 08V194 (◆). The virus was passages 5 times and the supernatant was titrated. Values represent mean ± SD of three titrations.

Virus sequencing after passaging virus in PK15$^{Sn\text{-}CD163}$ cells

As most virus strains grow better on PK15$^{Sn\text{-}CD163}$ cells, this cell line was more interesting to grow vaccine virus than CHO$^{Sn\text{-}CD163}$ cells. To produce vaccine virus for inactivated vaccines mutation of structural proteins should not occur. Therefore, PK15$^{Sn\text{-}CD163}$ grown virus was sequenced and compared to the sequence of the original virus. VR-2332 and 07V063 grown on PK15$^{Sn\text{-}CD163}$ cells showed no differences after 5 passages compared to the original virus strain. PK15$^{Sn\text{-}CD163}$ grown LV showed 1 amino acid (aa), 08V194 2 aa and 08V204 3 aa differences compared to macrophage grown virus. For LV the aa change was in ORF4 (N37D). The aa differences of 08V194 were located in ORF2 (E73D) and in ORF5 (N37S). For 08V204 the aa differences were situated in ORF2 (N37D), ORF4 (I121V) and ORF5 (N37D).

4.1.5 DISCUSSION

PRRSV replicates efficiently in *ex vivo* cultivated primary macrophages, which are the natural host cells. For vaccine virus production however, this cell type cannot be used, because of batch variation, risk of contamination with other pathogens and high production costs. PRRSV susceptible cell lines, such as the African green monkey derived cell lines, like Marc-145, have the potential to overcome problems associated with the use of primary macrophages, such as up-scaling and safety. However, PRRSV infects Marc-145 cells via a different entry pathway compared to macrophages, which results in adaptation of the virus for growth on Marc-145 cells (Collins et al., 1992).

Several non-permissive cells transfected with RNA of PRRSV could produce infectious virus. It is therefore suggested that the susceptibility of cells for PRRSV infection is determined by membrane-associated components (Kreutz, 1998). In a previous study it was shown that non-permissive cells transiently transfected with Sn only sustained internalization, but not infection (Vanderheijden et al., 2003). Non-permissive cells transiently transfected with CD163 may allow a low level of infection depending on the cell type used (Calvert et al., 2007). Co-expression of both Sn and scavenger receptor CD163 are needed for an efficient PRRSV infection (Van Gorp et al., 2008). It was shown that a virus titer ranging between $10^{2.4}$ and $10^{5.5}$ $TCID_{50}$/mL could be obtained upon PRRSV infection of cells transiently transfected with recombinant Sn and CD163 (Van Gorp et al., 2008). Since only a part of the cells were transfected upon transient transfection, it was expected that the virus titer would be higher if stably transfected cell lines could be used. In this study, cell lines that express both recombinant Sn and CD163 ($CHO^{Sn\text{-}CD163}$ and $PK15^{Sn\text{-}CD163}$) were generated, as both receptors are involved in infection of the natural host cell, the macrophage (Delputte et al., 2005; Van Gorp et al., 2008). The constructed $CHO^{Sn\text{-}CD163}$ and $PK15^{Sn\text{-}CD163}$ cell lines were first analyzed for their PRRSV susceptibility. They were both susceptible for PRRSV, because LV and VR-2332 virus attachment, internalization, disassembly and infection occurred in both cell lines.

When analyzing the PRRSV susceptibility, it was shown that primary infection of the cell lines was more efficient with Marc-145 grown virus than macrophage grown virus. The infection rate of macrophage grown virus was very low, most likely because binding and internalization of macrophage grown virus particles into the cell lines was not efficient. The interaction of macrophage grown PRRSV with Sn, the receptor mediating binding to and internalization into the cells, is probably not efficient. This can be due to sialic acids present

on the cells interfering with Sn, resulting in competition with sialic acid on PRRSV. This hypothesis is based on the observation that CD33, also a member of the sialoadhesin family, transfected in COS cells were not able to bind to red blood cells, containing sialic acid, unless the COS cells were first treated with sialidase to remove endogenous ligands (Ducreux et al., 2009; Freeman et al., 1995). Also CHO^{Sn} cells showed no binding of red blood cells unless they were treated with neuraminidase (Delputte et al., 2007b).

To investigate if neuraminidase could improve macrophage grown PRRSV infection, the CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells were first treated with *Vibrio cholerae* neuraminidase (Roche) to remove potential *cis*-acting sialic acids that could interfere with the sialic acid binding capacity of Sn (Delputte et al., 2007b). This resulted in an increased amount of macrophage grown virus infected cells. These results suggest that the low virus titers are related with a low binding capacity of PRRSV to Sn, because of sialic acid present on the CHO$^{Sn-CD163}$ and PK15$^{Sn-CD163}$ cells. Marc-145 grown PRRSV will most likely also contain sialic acids that can interfere with Sn. Our hypothesis is that also the production of Marc-145 grown virus can be improved by neuraminidase treatment of the cells and needs to be further investigated.

However, using an expensive product like *Vibrio cholerae* neuraminidase is not ideal for vaccine production. To avoid this problem, virus was grown via several passages on the cell lines. Normally, titers of 10^5-10^7 $TCID_{50}$/mL can be obtained on Marc-145 cells after 5 to 7 passages (Benfield et al., 1992; Kim et al., 1993). The results show that the PK15$^{Sn-CD163}$ cells give similar results as a recent macrophage cell line transfected with CD163 (Lee et al., 2009). In addition, the PK15$^{Sn-CD163}$ cells also express Sn, which is important to facilitate virus entry. The macrophage cell line transfected with CD163 also expressed Sn (Lee et al., 2009), which confirms that both receptors, Sn and CD163, are important for an efficient virus production. Virus growth on PK15$^{Sn-CD163}$ cells resulted in higher titers than growth on CHO$^{Sn-CD163}$ cells and the virus titer achieved on PK15$^{Sn-CD163}$ cells was equal to the titer on Marc-145 cells, which makes the PK15$^{Sn-CD163}$ cell line an interesting tool for virus production.

It is reported that due to adaptation of PRRSV to Marc-145 cells, mutations in non-structural, but also structural viral proteins may occur (Allende et al., 2000; Indik et al., 2000; Zhou et al., 2009). For the production of an inactivated virus vaccine, mutations in ORFs encoding viral structural proteins are not desired, since this can influence the induction of a virus neutralizing antibody response. The virus grown on PK15$^{Sn-CD163}$ cells is expected to show less mutation than after growth on Marc-145 cells, since the PK15$^{Sn-CD163}$ cells express Sn and

CD163, two receptors important in the entry of the virus in macrophages. To investigate if virus grown on PK15$^{Sn\text{-}CD163}$ cells showed mutation in ORFs encoding viral structural proteins, ORF2a, 3, 4, 5, 6 and 7 were sequenced. 07V063 and VR-2332 grown on PK15$^{Sn\text{-}CD163}$ cells showed a 100% aa identity with 07V063 grown on macrophages and VR-2332 grown on Marc-145 cells. LV grown on PK15$^{Sn\text{-}CD163}$ cells had 1 aa changed in ORF 4 compared to macrophage grown LV, which resulted in a loss of a putative glycosylation site. The mutation was not situated in a known neutralizing epitope (Meulenberg et al., 1997; Oleksiewicz et al., 2001). PK15$^{Sn\text{-}CD163}$ grown 08V194 had 2 aa differences compared to macrophage grown 08V194. There was 1 aa changed in ORF5. This resulted not in a loss of a glycosylation site, but the glycosylation site moved to another place. The mutation was not situated in a known neutralizing epitope (Plagemann, 2006). The second aa change was located in ORF2a, however a change from E to D is supposed to have no effect on the protein structure since those aa are similar. For 08V204 grown on PK15$^{Sn\text{-}CD163}$ cells, there was 1 aa changed in ORF2a, 1 aa in ORF4 and 1 aa in ORF5. The mutation in ORF4, however was a change from I to V, which are comparable aa and will not have an influence on the protein structure. The mutation in ORF5 results in a loss of a putative glycosylation site, but is not located in a known neutralizing epitope (Plagemann, 2006). The mutation in ORF5 of 08V194 and 08V204 are both on position 37. This position varies among different PRRSV strains and is a not well conserved glycosylation site (Prieto et al., 2009). These results indicate that the PK15$^{Sn\text{-}CD163}$ cells are useful for production of vaccine virus, but each strain should be investigated for aa changes in the structural proteins before use in inactivated vaccine production. Experiments are ongoing to test the immunogenicity of inactivated PRRSV grown on PK15$^{Sn\text{-}CD163}$ cells and the effect of the minor aa changes on the induction of a protective immunity towards challenge virus.

The observation that all tested strains grow well on the PK15$^{Sn\text{-}CD163}$ cells also suggests that these cells might be useful for virus isolation. Currently Marc-145 cells are used for diagnostics, but it has been shown that not all PRRSV strains can be detected on those cells (de Abin et al., 2009). Macrophages, the natural host cell of PRRSV, are more efficient for virus isolation (de Abin et al., 2009). The difference in isolation efficiency between Marc-145 cells and macrophages is suggesting that another receptor next to CD163 is involved in infection as Marc-145 cells and macrophages both express CD163 (Calvert et al., 2007; Van Gorp et al., 2008). The PK15$^{Sn\text{-}CD163}$ cells can be useful for virus isolation, because of the expression of both CD163 and Sn, but needs to be further investigated.

4.1.6 ACKNOWLEDGEMENTS

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4.2 COMPARISON OF THE EFFICACY OF INACTIVATED PRRSV VACCINE VIRUS GROWN ON MARC-145 OR PK15$^{Sn\text{-}CD163}$ CELLS

In preparation

4.2.1 SUMMARY

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. In the field, both killed virus (KV) and modified live virus (MLV) vaccines are used to control the disease. The immune response induced by current commercial KV vaccines in naïve pigs does not offer sufficient protection. This can be due to PRRSV strain variability, the inactivation method used and the use of Marc-145 cells for the production of vaccine virus. Adaptation of the virus for growth on Marc-145 cells is necessary, which may cause unwanted mutations in genes encoding important envelope glycoproteins. Therefore, a $PK15^{Sn\text{-}CD163}$ cell line expressing porcine sialoadhesin (Sn) and porcine CD163, two important receptors for entry and infection of macrophages, the natural host cells of PRRSV, was developed for PRRSV production. In this study, an experimental binary ethyleneimine (BEI) inactivated PRRSV vaccine based on a recent PRRSV isolate was used. The protection provided by the immune response induced by a KV vaccine based on Marc-145 grown virus was compared to that induced by a KV vaccine based on $PK15^{Sn\text{-}CD163}$ grown virus. Vaccination of naïve pigs with BEI-inactivated, Marc-145 as well as $PK15^{Sn\text{-}CD163}$ grown virus induced a virus-specific antibody response. Vaccination with BEI-inactivated, Marc-145 grown virus induced a virus-neutralizing antibody response, while vaccination with BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown virus only primed a virus-neutralizing antibody response. Vaccination with a high dose of BEI-inactivated, Marc-145 grown virus (10^9 $TCID_{50}$) resulted in a significant reduction of viremia upon challenge, while a low dose of BEI-inactivated, Marc-145 grown virus (10^8 $TCID_{50}$) did not, indicating that the vaccine dose of 10^8 $TCID_{50}$ was too low. Both vaccinations based on BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown virus (10^9 $TCID_{50}$ and 10^8 $TCID_{50}$) resulted in a clear reduction of viremia. If the vaccine virus was produced in $PK15^{Sn\text{-}CD163}$ cells, a vaccine dose of 10^8 $TCID_{50}$ was sufficient to obtain a reduction in viremia. To obtain a similar reduction of viremia using Marc-145 grown vaccine virus, a vaccine dose of 10^9 $TCID_{50}$ was necessary.

4.2.2 INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for reproductive failure in sows and is associated with the porcine respiratory disease complex (PRDC) in pigs of all ages and is the major economic viral pig disease worldwide (Christianson et al., 1993; Lager and Mengeling, 1995; Mengeling et al., 1994; Neumann et al., 2005; Terpstra et al., 1991). There are several commercial vaccines, but their efficacy and safety is not always sufficient. Modified live virus (MLV) 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 about safety, as the vaccine strain may spread and revert to virulent virus (Dewey et al., 1999; Mengeling et al., 1999; Nielsen et al., 2001; Nielsen et al., 1997). Killed virus (KV) vaccines are safe to use, but the commercial KV vaccines do not provide sufficient protection (Nielsen et al., 1997; Nilubol et al., 2004; Zuckermann et al., 2007).

The production process of a KV vaccine can be subcategorized in (i) the production of vaccine virus, (ii) inactivation of vaccine virus and (iii) formulation of the KV vaccine. PRRSV vaccine virus is currently produced in Marc-145 cells, because a cell line has the potential to overcome problems associated with the use of primary macrophages, the natural host cell of PRRSV, such as up-scaling and safety. Adaptation of the virus strain for growth on Marc-145 cells is necessary, since the entry and maybe also the replication in Marc-145 cells is different as in macrophages (Collins et al., 1992; Delputte et al., 2004). Due to this adaptation, mutations in open reading frames (ORFs) encoding viral proteins can occur as described for VR-2332 passaged in MA-104, where mutations in non-structural proteins nsp1β, nsp2 and nsp10, but also mutations in structural proteins GP3, GP5 and M occur (Allende et al., 2000). For the Chinese strain HUN4 and the Czech strain V502, it is shown that after passaging the strains in Marc-145 cells, mutations in ORF5 occur (Indik et al., 2000; Zhou et al., 2009). Mutations in structural viral proteins can result in an ineffective humoral immune response towards field strains after vaccination. To avoid mutations in viral structural proteins after growth on a cell line, a PRRSV susceptible cell line mimicking a natural entry pathway by expressing two important receptors for PRRSV entry and infection on macrophages, porcine sialoadhesin (Sn) and porcine CD163, is introduced for PRRSV production (Delrue et al., 2010). Sn is a receptor that mediates PRRSV attachment to and internalization into macrophages (Delputte et al., 2005; Vanderheijden et al., 2003) and

CD163 is suggested to be involved in virus uncoating in macrophages (Van Gorp et al., 2008). Expression of both receptors are shown to be sufficient for PRRSV infection of non-permissive cells (Van Gorp et al., 2008).

The inactivation of a virus for KV vaccine development has to meet two major goals: (i) the virus has to be completely inactivated to avoid viral infection and (ii) the neutralizing epitopes need to be preserved in order to enable the induction of a proper virus-neutralizing (VN) antibody response. The current incomplete protection of a KV vaccine against PRRSV infection may be due to the inactivation procedure used to inactivate the virus, which can affect the viral proteins and might destruct the neutralizing epitopes (Delrue et al., 2009). PRRSV inactivation with binary ethyleneimine (BEI) is shown to inactivate PRRSV with preservation of the entry-associated domains (Delrue et al., 2009). Viral entry-associated domains are most likely important for 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). Vanhee et al. (2009) showed that vaccination with Marc-145 grown Lelystad virus (LV) inactivated with BEI in an oil-in-water adjuvant can induce a virus-specific and a VN antibody response, resulting in a significant reduction of viremia after challenge (Vanhee et al., 2009).

In this study, the efficacy of a BEI-inactivated vaccine based on a recent PRRSV strain, 07V063, grown on $PK15^{Sn\text{-}CD163}$ cells is compared to Marc-145 grown vaccine virus to investigate if the experimental inactivated PRRSV vaccine can be improved if the vaccine virus is produced in $PK15^{Sn\text{-}CD163}$ cells instead of Marc-145 cells. $PK15^{Sn\text{-}CD163}$ cells are expressing two important macrophage receptors, thereby mimicking the entry and infection pathway in macrophages and probably avoiding mutations in viral structural proteins.

4.2.3 MATERIALS AND METHODS

Cells and viruses

A recent Belgian PRRSV field strain (07V063) was isolated in 2007 from aborted fetuses, coming from sows that were frequently vaccinated with a European attenuated PRRSV vaccine. The virus produced in Marc-145 cells or $PK15^{Sn\text{-}CD163}$ cells, was used for vaccination. A fifth passage of this strain in porcine alveolar macrophages (PAM), derived from gnotobiotic piglets, was used to challenge the animals. Marc-145 cells were cultivated in minimum Eagle's medium (MEM) with 5% fetal bovine serum (FBS), 2 mM L-glutamine and

a mixture of antibiotics. PK15$^{Sn-CD163}$ cells were cultivated in MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate and a mixture of antibiotics. The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. Marc-145 cells and PK15$^{Sn-CD163}$ cells were used for virus production. Virus internalization was investigated in PAM cultivated in RPMI-1640 with 10% FBS, 2 mM L-glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate and a mixture of antibiotics for 24 hours. PAM were also used for virus titrations.

Virus sequencing of macrophage grown, Marc-145 grown and PK15$^{Sn-CD163}$ grown 07V063

RNA was extracted from PRRSV using an 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'-cggcccaittccatccigag-3') and ORF4-REV (5'-cattcagctcgcataicgtcaag-3'), ORF5-FW2 (5'-tgcticatttcitgacacc-3') and ORF5-REV1 (5'-accttaagigcitatatc-3'), ORF6FW (5'-taccaactttcttctggac-3') and ORF6REV (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 Biosystems, Foster City, USA) and PRRSV primers. Cycle sequencing reaction products were purified by ethanol precipitation and separated on an ABI Genetic 310 (Applied Biosystems, Foster City, USA).

The sequences were analyzed and compiled by BlastN and BlastP (www.ncbi.nlm.nih.gov), and Sixframe, ClustalW, Align (workbench.sdsc.edu). The Genbank accession number of 07V063 is [Genbank:GU737264]

PRRSV concentration and semi-purification

The Belgian PRRSV field strain was grown on Marc-145 cells or PK15$^{Sn-CD163}$ cells. The supernatant containing the virus was purified as described by Delputte et al., 2004, this with some modifications to allow purification of larger quantities of virus. Cell culture supernatant containing virus was first filtrated through a 0.45 µm filter and then ultra-centrifuged for 2

hours at 112 000 g with a rotor type 35 at 4°C (Beckmann Coulter) to pellet the virus. The resuspended virus pellet was centrifuged 10 minutes at 16 200 g (Heraeus fresco) to remove cell debris and large aggregates and the supernatant was ultra-centrifuged through a 30% sucrose cushion for 3 hours at 100 000 g with a SW 41 Ti rotor at 4°C (Beckmann Coulter). Finally, the virus pellet was resuspended in 1 mL PBS after incubating it with PBS for 1 hour on ice.

PRRSV inactivation

Inactivation of PRRSV with binary ethyleneimine (BEI) was performed as described by Bahnemann (Bahnemann, 1990). A 0.1 M stock of BEI was prepared by cyclization of 2-bromoethylamine in 0.175 M NaOH for 1 hour at 37°C. Virus was inactivated by incubation with 1 mM BEI for 24 hours at 37°C. Afterwards, BEI was neutralized by incubation with 0.1 mM sodium thiosulphate for 2 hours at 37°C. Inactivated virus was stored at -70°C.

Analysis of virus inactivation

To confirm that all virus was completely inactivated, a complete vaccine dose (10^9 $TCID_{50}$/mL, titrated on macrophages) was inactivated and used for inoculation of Marc-145 cells, followed by two passages. The Marc-145 cells were investigated every week for CPE and cells were stained by immunoperoxidase monolayer assay (IPMA) with monoclonal antibody P3/27 against the PRRSV nucleocapsid protein to detect infected cells (Wieczorek-Krohmer et al., 1996).

Analysis of internalization of inactivated PRRSV by immunofluorescence staining

Macrophages were inoculated with untreated or BEI-inactivated PRRSV (moi 2) for 1 hour at 37°C, fixed with methanol at -20°C and stained as described by Delputte et al., 2004. Briefly, the capsid protein was stained with a primary antibody P3/27 (Wieczorek-Krohmer et al., 1996) and a secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-mouse immunoglobulins (Molecular Probes). Confocal analysis was performed using a TCS SP2 laser scanning spectrum confocal system (Leica Microsystems GmbH, Heidelberg, Germany) to determine the amount of internalized PRRSV particles.

Analysis of the viral antigen load in inactivated PRRSV vaccines by SDS-PAGE and Western blotting

SDS-PAGE and Western blotting of viral nucleocapsid protein was performed as described by Delputte et al., 2007a. The membrane was stained for 1 hour with a primary mouse monoclonal antibody against the nucleocapsid protein N (P3/27) (Wieczorek-Krohmer et al., 1996).

Experimental design

Piglets derived from a PRRS-negative farm were used. The piglets were randomly divided into five groups. As an adjuvant, an oil-in-water (o/w) diluent that is used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky (Fort Dodge Animal Health, Kelmis, Belgium) was used, further called o/w Suvaxyn. A first group (group A, n=12) was a mock-vaccinated control group and received 1 mL RPMI in 1 mL o/w Suvaxyn at 6 and 10 weeks of age. The other four groups were vaccinated twice intramuscularly at 6 (primo vaccination) and 10 (booster vaccination) weeks of age. Pigs in group B (n=12) were vaccinated with 1 mL BEI-inactivated, Marc-145 grown 07V063 (10^9 $TCID_{50}$/mL, titrated on macrophages) in 1 mL o/w Suvaxyn. Pigs in group C (n=6) received 1 mL BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 07V063 (10^9 $TCID_{50}$/mL, titrated on macrophages) in 1 mL o/w Suvaxyn. Pigs in group D (n=6) were vaccinated with 1 mL BEI-inactivated, Marc-145 grown 07V063 (10^8 $TCID_{50}$/mL, titrated on macrophages) in 1 mL o/w Suvaxyn. Pigs in group E (n=5) received 1 mL BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 07V063 (10^8 $TCID_{50}$/mL, titrated on macrophages) in 1 mL o/w Suvaxyn. Four weeks after booster vaccination, the pigs were challenged intranasally with 10^6 $TCID_{50}$ macrophage grown 07V063. Blood was taken weekly after vaccination and at 1, 3, 5, 7, 10, 14, 21 and 28 days post challenge. Serum was collected and stored at -70°C.

Virus-specific antibody detection

PRRSV-specific serum antibody titers were determined by IPMA as described by Labarque et al. (2000). Briefly, fixed Marc-145 grown 07V063 infected Marc-145 cells were stained with 10-fold dilution series of serum and secondary antibody peroxidase labeled goat anti-mouse immunoglobulin (Ig) (DakoA/S, Glostrup, Denmark). Afterwards, 3-amino-9-ethylcarbazole (AEC) substrate (Sigma) was added and the cells were analyzed with a light microscope (Olympus Optical Co., Hamburg, Germany). The IPMA antibody titer was expressed as the reciprocal of the last dilution that resulted in a positive reaction.

Virus-neutralizing (VN) antibody detection

VN antibody titers were detected by a seroneutralization (SN) test with Marc-145 grown 07V063 on Marc-145 cells (Labarque et al., 2000). Briefly, a 2-fold dilution series of serum was mixed with an equal volume of PRRSV ($2x10^3$ $TCID_{50}$/mL) and incubated for 1 hour at 37°C. The serum-virus mixture was then mixed with Marc-145 cells and seeded in a 96-well plate. The cells were checked for CPE at 10 days post inoculation. The VN antibody titer was defined as the reciprocal of the highest dilution that inhibited CPE in 50% of the inoculated wells.

Detection of viremia

Virus titers in serum were determined by virus titration on 24 hours cultivated alveolar macrophages following the standard procedure (Labarque et al., 2000). After 3 days incubation at 37°C, the occurrence of cytopathic effect (CPE) was investigated. Macrophages were fixed at 3 days post inoculation (dpi) and an immunoperoxidase staining with monoclonal antibody P3/27 against the nucleocapsid protein of PRRSV was performed to determine infected cells. The 50% tissue culture infective dose ($TCID_{50}$/mL) was calculated.

Statistical analysis

Antibody and virus titers were analyzed by Kruskall-Wallis test, followed by Dunn's multiple comparisons test to determine differences between 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).

4.2.4 RESULTS

Virus sequencing

To determine if adaptation to both Marc-145 and $PK15^{Sn\text{-}CD163}$ cell lines resulted in mutations in the viral structural proteins after growth on Marc-145 and $PK15^{Sn\text{-}CD163}$ cells, ORF2-3-4-5-6 and 7 of Marc-145 grown and $PK15^{Sn\text{-}CD163}$ grown 07V063 were compared with original macrophage grown 07V063. The ORFs of $PK15^{Sn\text{-}CD163}$ grown as well as Marc-145 grown 07V063 were 100% identical to macrophage grown 07V063.

Control of virus inactivation, virus internalization and antigen load

PRRSV that was either produced in Marc-145 cells or PK15$^{Sn-CD163}$ cells was inactivated with BEI. To confirm the complete inactivation of the virus, a vaccine dose was passaged in Marc-145 cells, followed by immunostaining for the nucleocapsid protein of PRRSV to detect infected cells. Cells inoculated with BEI-inactivated virus did not show CPE or positive nucleocapsid staining, while CPE as well as nucleocapsid staining occurred in cells inoculated with untreated virus. Further, the conservation of the entry-associated domains after inactivation was investigated. Therefore, macrophages were inoculated with inactivated and untreated virus to confirm that inactivated virus could still be bound and internalized by macrophages. This experiment showed no differences between BEI-inactivated and untreated virus, confirming that the entry-associated domains were preserved after inactivation (Fig. 4.6A). The viral antigen load for all vaccines was tested by Western blotting. The vaccines consisting of 10^9 $TCID_{50}$ PRRSV/mL contained similar amounts of antigens and was higher than that of the vaccines with a vaccine dose of 10^8 $TCID_{50}$/mL (Fig. 4.6B).

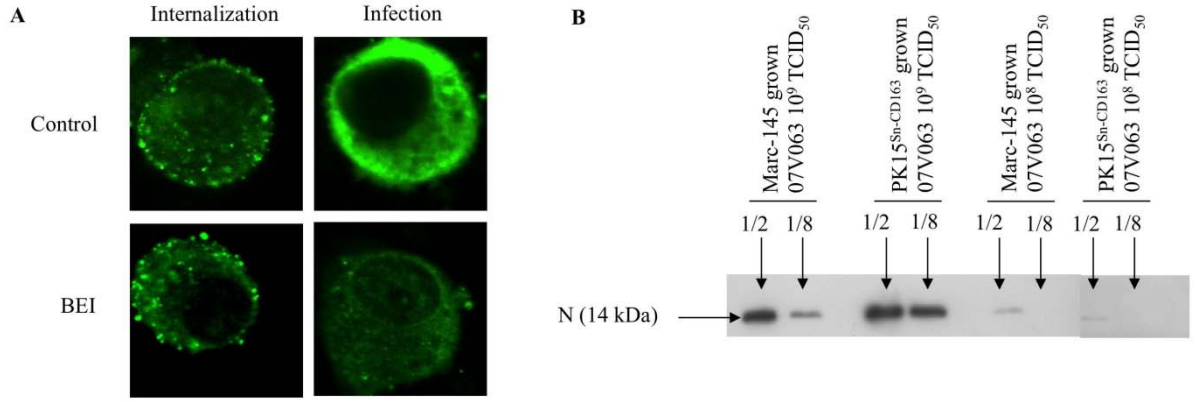

Figure 4.6. Controls of the BEI-inactivated vaccines. A) Internalization and infection of untreated control 07V063 and BEI-inactivated 07V063 in macrophages. B) WB analysis of the antigen load of the vaccines.

Virus-specific antibody response

In the mock-vaccinated control group (group A) no virus-specific antibodies were detected by IPMA before challenge (Fig. 4.7). The first virus-specific antibodies in pigs in group A were detected starting from 7 days post challenge (9/12) until the end of the experiment (four weeks post challenge). All pigs vaccinated with BEI-inactivated, Marc-145 grown virus (10^9 $TCID_{50}$) (group B), PK15$^{Sn-CD163}$ grown virus (10^9 $TCID_{50}$) (group C), Marc-145 grown virus (10^8 $TCID_{50}$) (group D) or PK15$^{Sn-CD163}$ grown virus (10^8 $TCID_{50}$) (group E) had virus-specific antibodies starting from one week post booster vaccination until the end of the experiment. The virus-specific antibody response was significantly higher than that of the

control group A, starting from one week post booster vaccination (group B, C and E) until 5 days (group C and E) or 10 days (group B) post challenge. The pigs in group D showed a significantly higher virus-specific antibody response than that of the control group starting from two weeks post booster vaccination until the end of the experiment (four weeks post challenge).

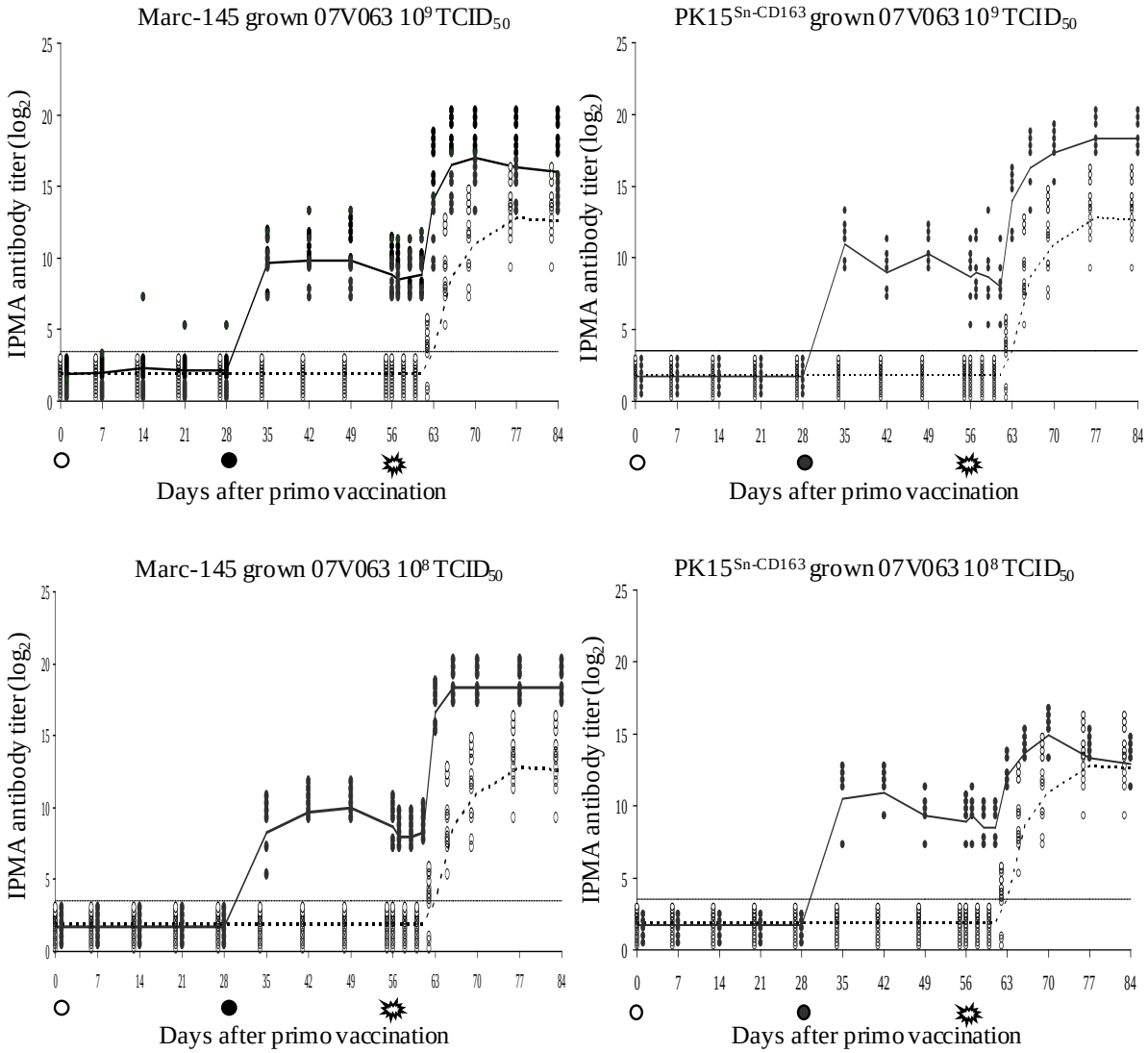

Figure 4.7. Virus-specific antibody titers after vaccination with BEI-inactivated PRRSV grown on Marc-145 and PK15$^{Sn-CD163}$ cells and after challenge.
Virus-specific antibodies were determined with an IPMA test. The IPMA titers of the animals from the control group are represented by open symbols, those from the vaccinated group by filled symbols. The dotted line gives the mean IPMA titer for the control group. The full line gives the mean for the vaccinated group. The dashed line gives the detection limit. ○ = primo vaccination, ● = booster vaccination, ✸ = challenge

Virus-neutralizing (VN) antibody response

VN antibodies were not detected before challenge in the pigs in the control group (group A) and in both groups vaccinated with 10^8 $TCID_{50}$ BEI-inactivated virus (group D and E), except

for two pigs in group D. One pig in group D had an SN antibody titer of 3 log_2 at one week post booster vaccination and one had an SN antibody titer of 1 log_2 at three weeks post booster vaccination (Fig. 4.8). Next to those two pigs, the VN antibodies appeared in the other pigs in group D starting from two weeks post challenge and had a mean titer of 2.5 log_2. The mean SN antibody titer of the pigs in group D was significantly higher than that of the control pigs starting from two weeks post challenge. All animals in the control group A remained negative during the experiment. In pigs vaccinated with 10^9 $TCID_{50}$ BEI-inactivated, Marc-145 grown virus (group B), the VN antibodies appeared starting from one week post booster vaccination (11/12) and had a mean SN antibody titer of 3.6 log_2 at one week post booster vaccination. Starting from two weeks post booster vaccination the mean SN antibody titer of the pigs in group B declined, but it increased again after challenge. The mean SN antibody titer was significantly higher than that of the control group starting from one week post booster vaccination until four weeks post challenge, except at 1 day post challenge. The pigs vaccinated with 10^9 $TCID_{50}$ BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown virus (group C), the VN antibodies appeared starting from two weeks post challenge (6/6) and had a mean SN antibody titer of 3.3 log_2 at that time point. The mean SN antibody titer was significantly higher than that of the control group starting from 14 days post challenge. In the pigs in group E, the SN antibodies were detected starting from 7 days post challenge (2/5) with a mean titer of 1.5 log_2 at that time point and increasing to 4.5 log_2 at the end of the experiment (four weeks post challenge). The mean SN antibody titer of the pigs in group E was significantly higher than that of the control pigs starting from one week post challenge until the end of the experiment.

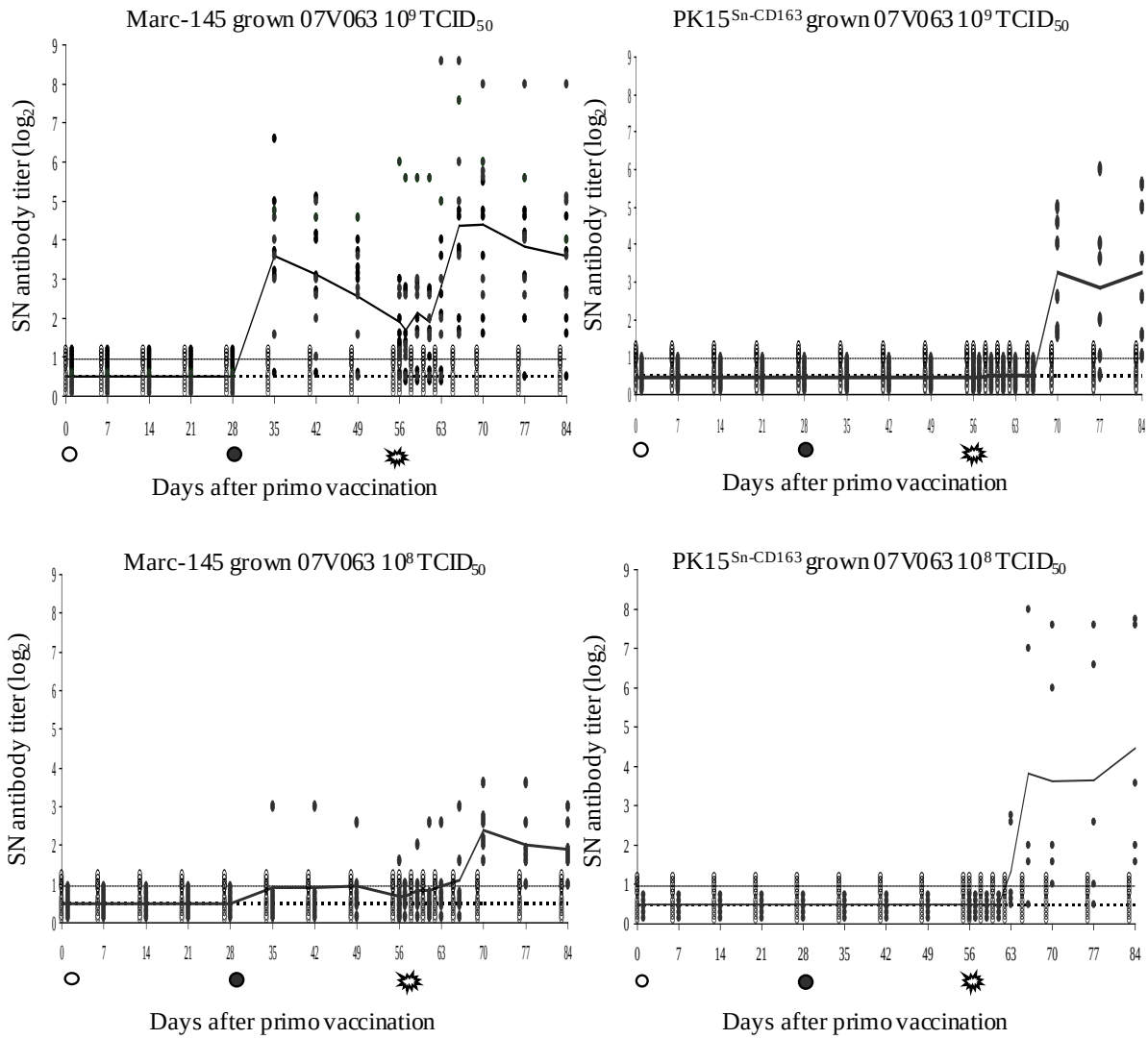

Figure 4.8. Virus-neutralizing antibody titers after vaccination with BEI-inactivated PRRSV grown on Marc-145 and PK15$^{Sn-CD163}$ cells and after challenge.
Virus-neutralizing antibodies were determined with an SN test. The SN titers of the animals from the control group are represented by open symbols, those from the vaccinated group by filled symbols. The dotted line gives the mean SN titer for the control group. The full line gives the mean for the vaccinated group. The dashed line gives the detection limit. ○ = primo vaccination, ● = booster vaccination, ✸ = challenge

Viremia

No virus was detected in serum at one week after each immunization, confirming that the vaccines were properly inactivated. Figure 4.9 shows the virus titers for each group post challenge. The pigs in the control group (group A) showed viremia starting from 1 day post challenge (7/12), the mean virus titer reached a maximum of 3.8 $\log_{10}$ $TCID_{50}$/mL at 10 days post challenge. All animals were virus-negative at 4 weeks post challenge, except for one pig. The pigs in groups B and C were viremic starting from 1 day post challenge (6/12 and 2/6 respectively). The mean virus titers in groups B and C were 3.1 $\log_{10}$ and 3.2 $\log_{10}$

$TCID_{50}$/mL respectively at 5 days post challenge. Starting from 10 days post challenge, however, the mean virus titers in groups B and C strongly decreased. In group B, all animals, except for three pigs were virus-negative at four weeks post challenge. In group C, only one pig was virus-positive at 10 days post challenge and all animals were virus-negative starting from two weeks post challenge, although some pigs showed a low virus titer at three weeks post challenge (4/6). The pigs in groups B and C showed a significant reduction of viremia starting from 10 days post challenge compared to the pigs in the control group. The pigs in group D showed viremia starting from 1 day post challenge (4/6), with a maximum mean virus titer at 7 days post challenge (3.6 $\log_{10}$ $TCID_{50}$/mL). In these pigs, viremia was still present at four weeks post challenge (2/6). The pigs in group D showed no significant reduction of viremia compared to the pigs in the control group. In group E, the pigs showed viremia starting from 1 day post challenge (4/5). At 5 days post challenge a maximum mean virus titer of 3.2 $\log_{10}$ $TCID_{50}$/mL was detected. Also in group E, the mean virus titer strongly decreased starting from 10 days post challenge, although viremia was detected in some pigs until the end of the experiment. Nevertheless, viremia in the pigs from group E was significantly reduced starting from 10 days post challenge compared to the pigs in the control group.

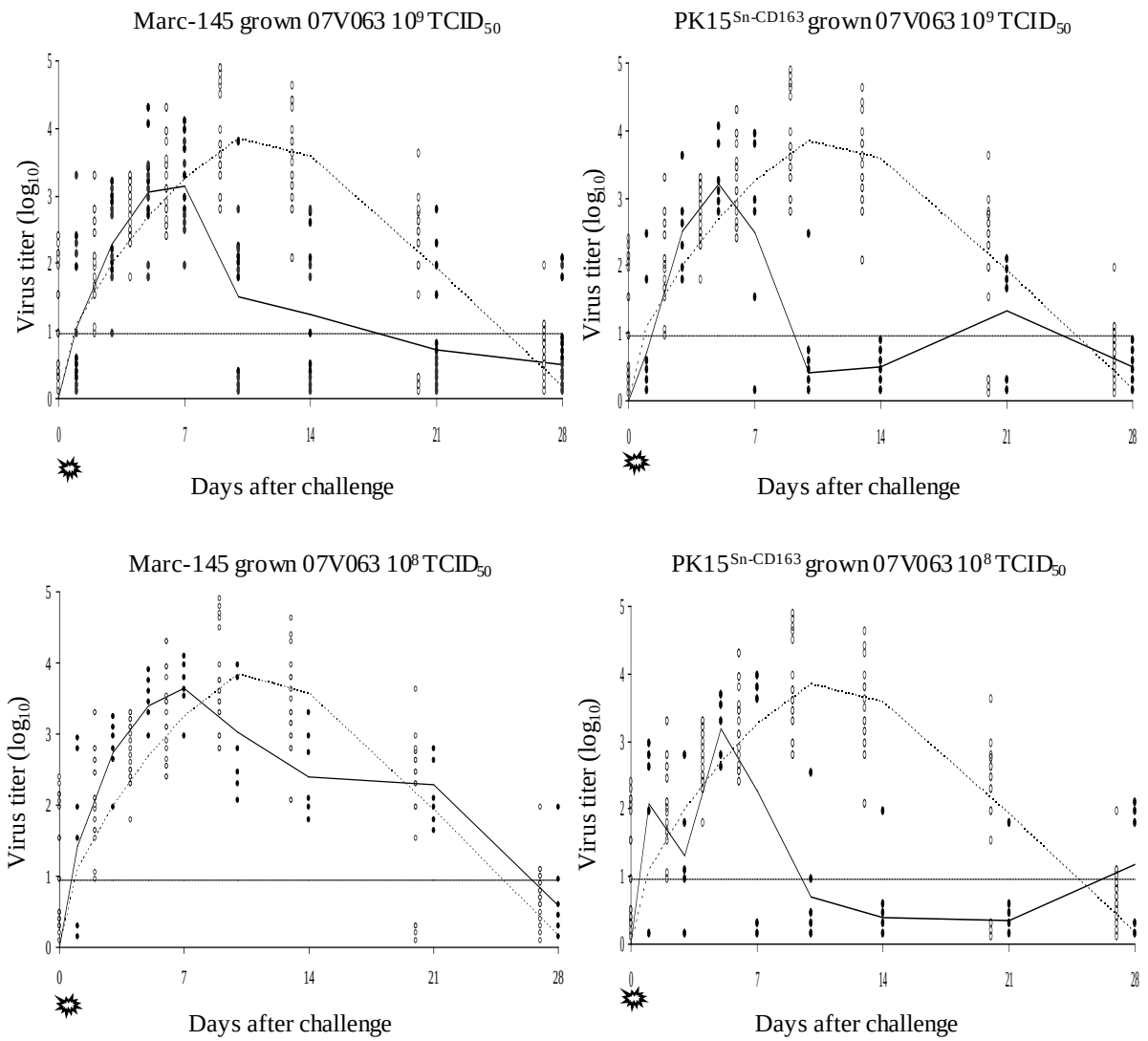

Figure 4.9. Virus titers after vaccination with BEI-inactivated PRRSV grown on Marc-145 and PK15$^{Sn-CD163}$ cells and after challenge.
Virus titers were determined by virus titration. The virus titers of the animals from the control group are represented by open symbols, those from the vaccinated group by filled symbols. The dotted line gives the mean virus titer for the control group. The full line gives the mean for the vaccinated group. The dashed line gives the detection limit. ✸ = challenge

4.2.5 DISCUSSION

PRRSV causes severe reproductive disorders in sows and boars and is associated with the porcine respiratory disease complex, leading to tremendous economic losses worldwide (Neumann et al., 2005). To control the disease, several commercial MLV and KV vaccines are available, but they both have some disadvantages. MLV vaccines are only effective if the circulating virus strain is closely related to the vaccine strain (Labarque et al., 2004) and MLV vaccines may cause some safety problems such as vaccine virus spread and reversion to virulence (Mengeling et al., 1999; Nielsen et al., 2001; Nielsen et al., 1997). Generally, KV

vaccines are safe to use, but their efficacy is questionable (Nielsen et al., 1997; Zuckermann et al., 2007). The poor efficacy of current available KV vaccines can be due to the inactivation procedure used, PRRSV strain variability and/or the use of Marc-145 cells to produce vaccine virus. Due to the use of some inactivation procedures for inactivating vaccine virus, there is a possibility that viral proteins and neutralizing epitopes necessary for the induction of a VN antibody response are modified or destroyed. Therefore, in this study the vaccine virus was inactivated with BEI following a protocol that preserves the entry-associated domains, which are most likely also important for the induction of a VN antibody response (Delrue et al., 2009). In the past, such an experimental BEI-inactivated PRRSV vaccine based on LV and an oil-in-water adjuvant has also been shown to be effective *in vivo* (Vanhee et al., 2009). In this study a recent circulating PRRSV strain was used for KV vaccine development instead of an LV-like strain, the currently used vaccine strain for European KV vaccines. Finally, the vaccine virus in this study produced in PK15$^{Sn\text{-}CD163}$ cells is compared to Marc-145 cells. Currently, Marc-145 cells are used for the production of vaccine virus, but adaptation for growth on this cell line is necessary, since the entry pathway and maybe also the replication in Marc-145 cells is different compared to macrophages, the natural host cells (Collins et al., 1992; Delputte et al., 2004). 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. Marc-145 cells express CD163 (Calvert et al., 2007), but not Sn (Vanderheijden et al., 2003), while macrophages express both Sn and CD163 (Van Gorp et al., 2008; Vanderheijden et al., 2003). The expression of both Sn and CD163 are shown to be sufficient for PRRSV infection of non-permissive cells (Van Gorp et al., 2008). Since the PK15$^{Sn\text{-}CD163}$ cells express two important macrophage receptors, Sn and CD163, involved in entry and infection of the natural host cell, it is expected that no or less mutations in the structural viral proteins will occur, since natural entry is mimicked. 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). For the recent PRRSV isolate used in this study, it is shown by sequencing that mutation of structural viral proteins do not occurs 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.

In this study, the efficacy of two doses of BEI-inactivated vaccines (10^9 and 10^8 $TCID_{50}$ titrated on macrophages) based on a recent PRRSV isolate grown on Marc-145 cells or PK15$^{Sn\text{-}CD163}$ cells was compared. The pigs of all vaccinated groups showed a virus-specific

antibody response, 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 a similar virus-specific antibody titer than the pigs in the control group.

Vaccination with BEI-inactivated, Marc-145 grown virus induced a significantly higher mean SN antibody titer than detected in serum from the pigs in the control group if a dose of 10^9 $TCID_{50}$ (group B) was used instead of 10^8 $TCID_{50}$ (group D), indicating that a dose of 10^8 $TCID_{50}$ is too low to induce a VN antibody response for the Marc-145 grown virus. In the pigs in the groups vaccinated with BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown virus, both the dose of 10^9 $TCID_{50}$ (group C) and 10^8 $TCID_{50}$ (group E), the SN antibody response was only detectable after challenge, but was strongly primed compared to the animals in the control group. The lack of detectable VN antibodies before challenge in the pigs in the groups vaccinated with $PK15^{Sn\text{-}CD163}$ grown virus 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. It is shown that the Belgian isolate we used in our experiment virus grown on $PK15^{Sn\text{-}CD163}$ cells or on Marc-145 cells is identical to the macrophage grown virus. However, a conformational change may lead to exposure of the neutralizing epitope on Marc-145 grown virus and make it more accessible for the induction of a VN antibody response before challenge. The difference between Marc-145 grown virus and $PK15^{Sn\text{-}CD163}$ grown virus can also lead to differences between VN antibodies induced by Marc-145 grown virus and $PK15^{Sn\text{-}CD163}$ grown virus, making the first detectable in the classical SN test and the others not. 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 during the whole test (10 days). When serum and virus are put together, VN antibodies will bind to the virus, but when this mixture is kept on the cells for 10 days, it is possible that VN antibodies with a low avidity that bind to the virus in the beginning via a weak binding, detach from the virus after a while. This problem can probably be solved by washing the serum-virus mixture away from the cells after 1 hour. 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. This hypothesis is based on the observation that the entry pathway in Marc-145 cells and macrophages is different (Delputte et al., 2004). Macrophages and $PK15^{Sn\text{-}CD163}$ cells express Sn and CD163, thereby stabilizing M/GP5, the ligand of Sn (Delputte et al., 2004; Delputte et al., 2007; Van Breedam et al., 2010), and stabilizing GP2/GP4, the ligand for

CD163 (Das et al., 2009). It is thus expected that Marc-145 grown virus will induce an antibody response against wild type (WT) GP2/GP4, since CD163 is present on Marc-145 cells and an antibody response against a modified GP5 which is not stabilized during entry, since Sn is not present on Marc-145 cells. Virus grown on PK15$^{Sn\text{-}CD163}$ cells is expected to induce an antibody response against WT GP2/GP4 and WT GP5, since Sn and CD163 are both present on those cells. WT GP5 is shown to have shielding glycans (Ansari et al., 2006), which might cause a decreased pool of GP5 neutralizing antibodies. Taken together, it is possible that there are no VN antibodies present before challenge in the serum of pigs vaccinated with BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown virus or they are not detectable with the currently used SN test. The optimization of this test and the pool of VN antibodies in all vaccinated groups will be studied in the future.

Reduction of viremia was observed in all pigs in the vaccinated groups starting from 10 days post challenge, except in the pigs in the group vaccinated with 10^8 $TCID_{50}$ BEI-inactivated, Marc-145 grown virus (group D), indicating that a vaccine dose of 10^8 $TCID_{50}$ is too low to induce the partial protection against infection. The pigs in group B, vaccinated with 10^9 $TCID_{50}$ BEI-inactivated, Marc-145 grown virus showed a reduction of viremia starting from 10 days post challenge, shortly after an increase of detectable SN antibody titers. When the pigs were vaccinated with 10^8 $TCID_{50}$ BEI-inactivated, Marc-145 grown virus (group D), the mean SN antibody titers remained low and there was no clear reduction of viremia. This is in accordance with the observation of the appearance of high SN antibody titers and the reduction of viremia are correlated as described by different authors (Albina et al., 1994; Labarque et al., 2003; Molitor et al., 1997; Vanhee et al., 2009). On the other hand, the pigs in group A (control) and group C (10^9 $TCID_{50}$ BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown virus) showed a reduction in viremia before VN antibodies were detectable, indicating that viremia can also be cleared in absence of VN antibodies (Diaz et al., 2006; Vanhee et al., 2009). The significant reduction of viremia in pigs in both groups vaccinated with PK15$^{Sn\text{-}CD163}$ grown virus, where no detectable VN antibodies are present before challenge, and the pigs in the group vaccinated with Marc-145 grown virus at a vaccine dose of 10^9 $TCID_{50}$, where detectable VN antibodies are present before challenge, can be explained in several ways. First, it is possible that VN antibodies are present before challenge in the pigs in the groups vaccinated with PK15$^{Sn\text{-}CD163}$ grown virus, but cannot be detected with the current used SN test. Second, it is possible that there are no VN antibodies present before challenge in the pigs in the groups vaccinated with PK15$^{Sn\text{-}CD163}$ grown virus, indicating that the VN antibodies before challenge are not important for the reduction of viremia after challenge, since

reduction of viremia is seen in the pigs in the group vaccinated with Marc-145 grown virus (10^9 $TCID_{50}$) with VN antibodies before challenge and in the pigs in both groups vaccinated with $PK15^{Sn\text{-}CD163}$ grown virus without VN antibodies before challenge.

In summary, BEI-inactivated vaccines based on 10^8 $TCID_{50}$ as well as 10^9 $TCID_{50}$ Marc-145 grown as well as $PK15^{Sn\text{-}CD163}$ grown virus induced a virus-specific antibody response. Vaccination with 10^9 $TCID_{50}$ BEI-inactivated, Marc-145 grown virus induced a VN antibody response, while vaccination with 10^8 $TCID_{50}$ BEI-inactivated, Marc-145 grown virus and 10^8 $TCID_{50}$ as well as 10^9 $TCID_{50}$ BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown virus only primed a VN antibody response. Vaccination with BEI-inactivated, Marc-145 grown virus (10^9 $TCID_{50}$) resulted in a significant reduction of viremia upon challenge, while BEI-inactivated, Marc-145 grown virus (10^8 $TCID_{50}$) did not, indicating that the vaccine dose of 10^8 $TCID_{50}$ was too low. Both vaccinations based on BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown virus (10^9 $TCID_{50}$ and 10^8 $TCID_{50}$) resulted in reduction of viremia. If the vaccine virus was produced in $PK15^{Sn\text{-}CD163}$ cells, a vaccine dose of 10^8 $TCID_{50}$ was sufficient to obtain a reduction in viremia. To obtain a similar reduction of viremia using Marc-145 grown vaccine virus, a vaccine dose of 10^9 $TCID_{50}$ was necessary.

4.2.6 ACKNOWLEDGEMENTS

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Chapter 5

GENERAL DISCUSSION

From an economic perspective, PRRSV is the most important viral pig disease worldwide (Neumann et al., 2005). This virus causes reproductive disorders in sows (Christianson et al., 1993; Mengeling et al., 1994; Terpstra et al., 1991), can temporarily reduce semen quality of boars (Prieto et al., 1996) and is associated with PRDC in pigs of all ages (Rossow et al., 1994). To control the disease in the field, currently several MLV and KV vaccines are used, but both types of vaccines have some disadvantages. MLV vaccines can induce a protective immunity, but only if the virus is genetically not too distant from the vaccine virus (Labarque et al., 2004; Labarque et al., 2003). In addition, MLV may cause some safety problems, such as transplacental spread of the vaccine virus (Dewey et al., 1999; Nielsen et al., 2002), temporary reduction of semen quality or virus shedding after vaccination of boars (Christopher-Hennings et al., 1997; Nielsen et al., 1997) and even reversion of vaccine virus to virulence (Nielsen et al., 1997). The currently available KV vaccines are safer in the field, but they insufficiently protect pigs against infection (Zuckermann et al., 2007). This can be due to PRRSV strain variability, destruction of viral neutralizing epitopes during the inactivation procedure or changes of the neutralizing epitopes during adaptation of the vaccine virus to cell lines. Therefore, a new, efficient and safe PRRSV vaccine is urgently needed. The major aim of this thesis was to develop an experimental inactivated PRRSV vaccine that is safe and effective. We preferred an inactivated vaccine, because it is rather easy to adjust the vaccine in a relatively short time to circulating strains in the field.

The production process of a KV vaccine can be divided into several sub-processes: virus strain selection, vaccine virus production, vaccine virus inactivation, adjuvant selection, determination of the vaccination dose and determination of the protective potential. This KV vaccine production process is presented in Figure 5.1.

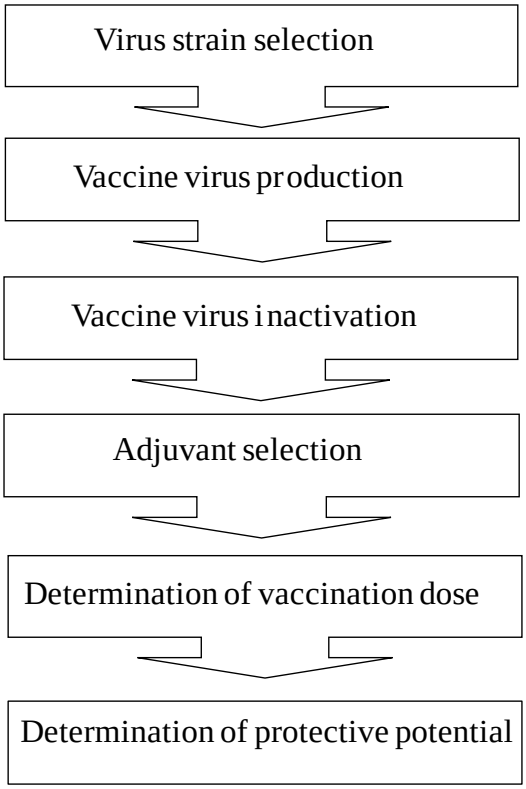

Figure 5.1. Production process of an inactivated virus vaccine

Virus strain selection

Due to PRRSV variability, it is useful to develop a farm-specific KV vaccine. In the present thesis, a recent PRRSV strain 07V063 was isolated during reproductive problems in a vaccinated herd in order to produce an inactivated PRRSV vaccine. Using 07V063 as a vaccine strain has the advantage that it is the circulating PRRSV strain on that farm. As a consequence, the vaccine strain is more related to the circulating strain than the LV-like strains currently used as European vaccine virus strains. For this study, a complete homologous situation was tested, thus vaccination and challenge with the same virus strain. This is of course not an ideal situation if for every farm another vaccine needs to be made. Therefore, it might be interesting to handle the situation of PRRSV like FMDV, where the outbreak strain is compared to several existing vaccine virus strains with for example a virus neutralization assay to decide which vaccine would be most effective (Paton et al., 2005).

Vaccine virus production

To develop a vaccine, a system to produce vaccine virus to a large scale is needed. For influenza virus, embryonated chicken eggs are often used for vaccine virus production. However, this has some disadvantages, such as risk for contamination and the presence of

allergenic components (James et al., 1998). Therefore, other systems like continuous cell lines were explored. Cell lines have the advantage to produce vaccine virus rapidly and on a large-scale (Genzel et al., 2006) and to reduce the risk of contamination. After testing several cell lines, Madin-Darby canine kidney (MDCK) and Vero cells were selected due to the high influenza virus yields that could be obtained (Govorkova et al., 1999; Liu et al., 2009). The continuous Vero cell line is recommended by the World Health Organization for vaccine production for human use. It has been used for the mass production of several viruses, such as rabies virus, poliovirus, enterovirus 71 and hantaan virus, in order to make vaccines (Choi et al., 2003; Frazzati-Gallina et al., 2001; Montagnon et al., 1984; Wu et al., 2004). Adaptation of the virus to Vero cells may induce unwanted genetic and antigenic changes which may reduce the efficacy of the virus upon vaccination. For instance, dengue virus production in Vero cells requires a strong adaptation to the host cell. To this end, MRC-5 cells were more suitable, because dengue virus remained stable during production (Liu et al., 2008). While chicken embryo cells (CEC) are often used for the production of Marek's disease virus (MDV) strains (Witter et al., 1995), permanent cell lines such as Vero cells also have been used, but the latter resulted in adaptation of the virus after several passages (Jaikumar et al., 2001). In a different approach, a permanent recombinant cell line, which supports efficient growth of both virulent and vaccine MDV strains, was generated (Schumacher et al., 2002). Quail muscle QM7 cells were constructed to constitutively express glycoprotein E, essential for MDV growth. These examples of the search for a satisfying virus production system illustrate that a general cell line for the production of viruses does not exist. The main cause for this variability is the delicate interplay between host cell and virus. A minor difference in the biology of a host cell, can cause viral adaptation, or even failure of viral attachment, internalization, disassembly, infection or replication.

In order to develop an inactivated PRRSV vaccine, a PRRSV susceptible cell line that produces high levels of vaccine virus is needed. *In vivo*, the virus infects a subpopulation of tissue macrophages (Duan et al., 1997a; Duan et al., 1997b; Labarque et al., 2000; Molitor et al., 1997). *In vitro*, PRRSV only replicates in primary pig macrophages (e.g. alveolar macrophages) (Wensvoort et al., 1991) and African green monkey kidney derived cells, such as Marc-145 (Kim et al., 1993; Wensvoort et al., 1991). Although virus production in primary macrophages would be ideal since they are the natural host cells, these cells cannot be used because of batch variation, risk of contamination with other pathogens and high production costs. Currently, vaccine virus is produced in Marc-145 cells, which have the potential to overcome problems associated with the use of primary macrophages, such as up-scaling and

costs (Kim et al., 1993). However, since virus entry and maybe also replication in Marc-145 cells is different compared to that in primary macrophages (Delputte et al., 2004), adaptation of the virus for growth on Marc-145 cells is necessary (Collins et al., 1992). Due to adaptation, mutations in non-structural, but also structural viral proteins may occur (Allende et al., 2000; Indik et al., 2000; Zhou et al., 2009), which may lead to an inefficient immune response. In the past, a continuous macrophage cell line was developed to cultivate PRRSV, but it was not permissive for the virus (Weingartl et al., 2002). This was due to the fact that the macrophage cell line did not express CD163, an important receptor for PRRSV infection (Lee et al., 2010). When CD163 was transfected in the macrophage cell line, which was already expressing Sn, a second important receptor for PRRSV infection, the cell line was susceptible for PRRSV. However, they did not investigate if mutations in structural viral proteins occurred after growth on the cell line (Lee et al., 2010). To avoid problems caused by mutations of structural viral proteins, we aimed in this thesis to create cell lines that recombinantly express receptors that mediate PRRSV entry and infection of macrophages, sialoadhesin (Sn) and CD163 (Chapter 4.1). Previously, it was shown that non-permissive cells transiently transfected with Sn only sustained internalization, but not infection (Vanderheijden et al., 2003). Non-permissive cells transiently transfected with CD163 may allow a low level of infection depending on the cell type used (Calvert et al., 2007). Co-expression of both Sn and scavenger receptor CD163 is needed for an efficient PRRSV infection (Van Gorp et al., 2008). Further research presented in this thesis showed that two stable cell lines, $CHO^{Sn\text{-}CD163}$ and $PK15^{Sn\text{-}CD163}$, were both susceptible for PRRSV, because LV and VR-2332 virus attachment, internalization, disassembly and infection occurred in both cell lines. Macrophage grown virus particles attached and internalized less efficiently than Marc-145 grown virus. This difference is possibly due to an inefficient interaction between Sn and sialic acid on the virus, because of interfering sialic acids on the cells competing with the virus. To test this hypothesis, an assay with red blood cells, which are known to have a high amount of membrane proteins containing sialic acid was used. Indeed, CHO^{Sn} cells showed no binding of red blood cells unless they were treated with neuraminidase, which supports our hypothesis (Delputte et al., 2007). When the cells were first treated with neuraminidase to remove competing sialic acids from the cells, the infection rate was higher. However, using an expensive product like *Vibrio cholerae* neuraminidase is not ideal for vaccine production. To overcome this problem, virus is grown via several passages in the cell lines. Not all tested PRRSV strains replicated in $CHO^{Sn\text{-}CD163}$ cells. Only the American prototype VR-2332 and one recent Belgian isolate (07V063) replicated well on

this cell line, while the European prototype LV and two other recent Belgian isolates (08V204 and 08V194) did not. In contrast, all 5 tested PRRSV strains replicated very well in the PK15$^{Sn\text{-}CD163}$ cells, with a virus yield equal to that obtained in Marc-145 cells. Virus growth on PK15$^{Sn\text{-}CD163}$ cells resulted in higher titers than growth on CHO$^{Sn\text{-}CD163}$ cells and the virus titer achieved on PK15$^{Sn\text{-}CD163}$ cells was equal than in Marc-145 cells, which makes the PK15$^{Sn\text{-}CD163}$ cell line an interesting tool for virus production. In this thesis a PRRSV susceptible cell line, PK15$^{Sn\text{-}CD163}$, was obtained that can be used for the production of vaccine virus, since all tested PRRSV strains could be produced in the cell line with titers similar than Marc-145 cells (Chapter 4.1). In the next step, the mutation rate of the 5 strains after growth on the PK15$^{Sn\text{-}CD163}$ cell line was investigated. For the production of a KV vaccine, we reasoned that mutations in ORFs encoding viral structural proteins should be avoided, since these mutations can influence the induction of a proper VN antibody response. Importantly, Marc-145 cells only express CD163 and lack Sn expression, resulting in the selection of PRRSV mutants that can enter the cell without using Sn. According to this hypothesis, the virus grown on PK15$^{Sn\text{-}CD163}$ cells is expected to show less mutations than after growth on Marc-145 cells, since the PK15$^{Sn\text{-}CD163}$ cells express Sn and CD163, two receptors important in the entry of the virus and infection of macrophages, mimicking the natural entry pathway. Analysis of the sequence of the structural proteins of original virus and virus grown for 5 passages in PK15$^{Sn\text{-}CD163}$ cells showed either no amino acid (aa) changes (VR-2332 and 07V063), one aa (LV), two aa (08V194) or three aa (08V204) changes. However, none of these changes were situated in known neutralizing epitopes (Meulenberg et al., 1997; Oleksiewicz et al., 2001; Plagemann, 2006).

Vaccine virus inactivation

An important criterium to develop a KV vaccine is that the virus should be completely inactivated to avoid outbreaks. The results in this thesis show that for every inactivation procedure optimal conditions to inactivate PRRSV could be obtained, except for AT-2. PRRSV did not appear to be sensitive for this product, although it has been reported to inactivate HIV properly (Chertova et al., 2003; Rossio et al., 1998). AT-2 modifies the free thiol groups of the internal viral proteins like the nucleocapsid of HIV-1, more specifically the zinc-finger motifs important for HIV-1 infection, leaving disulfide bridges of the glycoproteins in the virus envelope unaffected (Chertova et al., 2003; Williams et al., 2002). For PRRSV, the formation of homodimers of nucleocapsid proteins via disulfide bridges is important for virus infection, but as stated above, these disulfide bridges remain unaffected by

AT-2 (Wootton and Yoo, 2003). Therefore, a possible explanation could be that there are no free thiol groups in PRRSV that are important for infection, while the disulfide bridges that are important for infection, remain unaffected by AT-2. As AT-2 cannot inactivate PRRSV completely, it was not an option for inactivation of PRRSV for KV vaccine generation (Chapter 3).

A second criterium is that the viral neutralizing epitopes need to be preserved to be able to induce a protective immune response. Upon inactivation of the vaccine PRRS virus, the viral entry-associated domains need to remain preserved in order to generate a successful KV vaccine, since most neutralizing antibodies have an influence on attachment and entry of viruses into the host cell. This has been illustrated by several studies on ebolavirus, hepatitis C and SARS-CoV. For ebolavirus, it has been described that antibodies against the envelop glycoprotein (GP), important for virus attachment and entry, are able to neutralize the virus (Lee and Saphire, 2009). Also a neutralizing antibody, called CHB-5, against glycoprotein E2 of hepatitis C virus, is suggested to neutralize the virus by blocking the interaction between E2 and its cellular receptor CD81 leading to a block of infection (Owsianka et al., 2008). For SARS-CoV at least two kinds of neutralizing antibodies exist. Neutralizing antibodies against the receptor-binding domain of the spike protein prevent virus attachment, while neutralizing antibodies against an upstream region of the receptor-binding region prevent entry of the virus (Coughlin et al., 2009).

It has to be mentioned that neutralizing antibodies acting at the stage of disassembly also have been described, although it is rare. This is shown for parvovirus B19 (Ros et al., 2006). An external region of the capsid protein VP1, containing phospholipase activity necessary for virus infection, harbors neutralizing epitopes (Anderson et al., 1995). Binding of neutralizing antibodies to VP1 has been shown not to interfere with virus attachment to the host cell and the neutralizing antibodies only poorly bind to free virus (Saikawa et al., 1993). This indicates that the neutralizing antibodies only can bind to the neutralizing epitope on VP1 following a conformational change of VP1 caused by virus attachment to the cell, which might suggest that virus neutralization occurs after entry, possibly in the endosome (Ros et al., 2006). Antibodies against envelope protein E of West Nile virus (WNV) are suggested to neutralize the virus at the stage of pH-dependent fusion inside the cell (Diamond et al., 2008).

For PRRSV, it is shown that neutralizing antibodies can block the interaction of PRRSV with entry-receptor Sn (Delputte et al., 2004). Therefore, the viral entry-associated domains are most likely important for induction of a neutralizing antibody response. As a consequence, the hypothesis of the present study was that the most desirable inactivation methods are those that

are only affecting the viral genome, as they will most likely preserve the viral entry-associated domains. On the other hand, inactivation methods affecting the proteins of the virus, might damage viral proteins involved in virus entry, for example by cross-linking or denaturation. In such a case, the viral entry-associated domains are not preserved, making the method less interesting for the production of KV vaccines. In this thesis the effect of formaldehyde, glutaraldehyde, AT-2, pH changes, 37°C, UV irradiation, gamma irradiation and BEI on PRRSV were investigated.

Formaldehyde and glutaraldehyde have a similar effect on proteins and the viral genome, as they are both able to induce protein-protein cross-linking and RNA-protein cross-linking (Alderson, 1964; Cheung and Nimni, 1982; Fraenkel-Conrat, 1954). Cross-linking of viral neutralizing epitopes might interfere with the correct presentation of viral neutralizing epitopes to cells of the adaptive immune system. Experiments presented in this thesis showed that treatment of PRRSV with cross-linkers formaldehyde and glutaraldehyde did not preserve the viral entry-associated domains and are therefore not useful for inactivated PRRSV vaccine development (Chapter 3).

Another procedure used for viral inactivation consists of treatment with denaturing compounds. Procedures that have been documented to inactivate viruses by denaturation, are variation of pH and heat (Lelie et al., 1987; Schlegel et al., 2001; Weismiller et al., 1990). As is the case with protein cross-linking, denaturation of viral proteins may also destroy the viral neutralizing epitope(s). In this thesis, it was shown that inactivation by changing the pH did not preserve the viral entry-associated domains on PRRSV. This suggests that, at least in the case of PRRSV, inactivation by changing the pH is not sufficient to generate a proper KV vaccine (Chapter 3). A high temperature denaturates the proteins of the virus, but a modest increase of temperature until 37°C does not denaturate proteins, instead it degrades RNA (Dimmock, 1967; Fleming, 1971; Laude, 1981). *In vitro* infection assays and Western blot analysis showed that in the case of PRRSV the entry-associated domains were preserved after treatment at 37°C and that the virus did not replicate anymore (Chapter 3). Thus this method could be useful to inactivate PRRSV for the development of a KV vaccine.

Viruses can be inactivated by radiation, like UV irradiation and gamma irradiation. UV causes formation of pyrimidine dimers (uracil and thymine) between two adjacent pyrimidines (Miller and Plagemann, 1974; Sinha and Hader, 2002). The uracil dimers formed by UV irradiation inactivate the RNA molecule as a transcription template (Sauerbier and Hercules, 1978). More slowly, UV also causes structural modifications of the capsid proteins, resulting in large and small photoproducts (Miller and Plagemann, 1974). Experiments presented in this

thesis show that UV was an option to inactivate PRRSV for KV vaccine development, because the viral entry-associated domains were preserved (Chapter 3). Gamma irradiation mainly inactivates viruses by disrupting the viral genome (Grieb et al., 2002; Osborne et al., 2000), but free radicals that destroy the viral proteins can be formed. Gamma irradiation was also an option to inactivate PRRSV for KV vaccine generation, since the viral entry-associated domains were preserved (Chapter 3).

A final investigated procedure for inactivating viruses is by using an alkylating agent, like BEI. If a low concentration of BEI is used, the capsid is not alkylated, but BEI passes through the capsid and alkylates the genome (Bothner et al., 1998; Bothner et al., 1999; Broo et al., 2001; Lewis et al., 1998). BEI was an option to inactivate PRRSV for KV vaccine development, since the viral entry-associated domains were preserved, while completely inactivated (Chapter 3).

In conclusion, there are probably many methods that are suitable to inactivate a certain vaccine virus, but the success of these methods may vary depending on the particular virus and the conditions used (temperature, pH, concentration etc.). Nonetheless, successful methods to inactivate virus for vaccine development are best chosen from methods that mainly have an effect on the genome. Since formaldehyde, glutaraldehyde, pH and heat have an influence on protein level, it is possible that the neutralizing epitopes are modified by cross-linking (formaldehyde or glutaraldehyde) or denaturation (pH or temperature). In our opinion, UV irradiation, gamma irradiation and BEI are the most successful methods to inactivate a virus to create a KV vaccine, since these inactivation procedures mainly have an influence on genomic level. The results of this thesis confirmed that UV, gamma irradiation and BEI-inactivation of PRRSV preserved the viral entry-associated domains, while completely inactivating the virus (Chapter 3). These results suggest that UV, gamma irradiation and BEI can be useful to inactivate PRRSV for the generation of a KV vaccine. For inactivation of other viruses for KV development, it has to be considered that viral proteins can be damaged by UV photoproducts and free radicals formed by gamma irradiation. Although, the radiation target theory predicts that the radiation sensitivity of biomolecules depends on their mass (Osborne et al., 2000). Therefore viral genomes would be significantly more sensitive for damage than proteins.

Adjuvant selection

Since KV vaccines induce an insufficient immune response, the selection of an effective adjuvant is very important. Experimental vaccines based on inactivation procedures that preserve viral entry-associated domains, UV and BEI, based on Incomplete Freund's Adjuvant were investigated for their efficacy in pigs (Chapter 3). Vaccination with both UV- and BEI-inactivated Marc-145 grown LV with Incomplete Freund's Adjuvant strongly induced virus-specific antibodies in all animals and resulted in an earlier and strongly elevated VN antibody response after infection. The experimental vaccines were able to reduce viremia, starting from one week post infection. Moreover, viral clearance was systematically observed at earlier time points in vaccinated animals, compared to control animals, and reduction and/or clearance of viremia always coincided with the appearance of VN antibodies. The adjuvant was further optimized by Vanhee et al. (2009), with an oil-in-water adjuvant (an oil-in-water (o/w) diluent that is used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky (Fort Dodge Animal Health, Kelmis, Belgium)), giving the best results.

Determination of the vaccine dose and protective potential

The antigenic load also has an effect on the induction of a protective immunity. Therefore two different vaccination doses were investigated in the following vaccination study. BEI-inactivated PRRSV vaccines using Suvaxyn (oil-in-water (o/w) diluent that is used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky (Fort Dodge Animal Health, Kelmis, Belgium)) as adjuvant and based on 07V063 grown on Marc-145 cells and on $PK15^{Sn\text{-}CD163}$ cells were compared for their efficacy in pigs (Chapter 4.2). All optimized procedures from chapter 3 and chapter 4.1 were combined. In chapter 3, the inactivation procedure was optimized, with BEI giving the best results. In chapter 4.1, a PRRSV susceptible cell line was constructed ($PK15^{Sn\text{-}CD163}$), mimicking the entry pathway of the natural host cell, the macrophage, to avoid mutations in viral structural proteins (Delrue et al., 2010). In addition, a recent circulating PRRSV strain was used as a vaccine strain. No mutations of the structural viral proteins of 07V063 for growth on $PK15^{Sn\text{-}CD163}$ cells were observed (Chapter 4.1). Vaccination of naïve piglets with BEI-inactivated, Marc-145 as well as $PK15^{Sn\text{-}CD163}$ grown 07V063 induced a virus-specific antibody response before challenge and remained present post challenge. Commercial MLV vaccines are mostly able to induce a virus-specific antibody response before challenge (Diaz et al., 2006; Zuckermann et al., 2007), while virus-specific antibodies upon KV vaccination are reported to appear only after challenge (Misinzo et al., 2006; Zuckermann et al., 2007). However, with the optimized

inactivation protocol (Delrue et al., 2009) and adjuvant, it was possible to induce a virus-specific antibody response with an experimental BEI-inactivated Marc-145 grown LV in an oil-in-water adjuvant vaccine (Vanhee et al., 2009). VN antibodies appear delayed and are very weak in a PRRSV infection (Diaz et al., 2005; Labarque et al., 2000; Yoon et al., 1995). Some commercial MLV vaccines can induce a VN antibody response before challenge, although it is weak (Zuckermann et al., 2007), while others cannot (Diaz et al., 2006; Prieto et al., 2008). Commercial KV vaccines are not or almost not able to induce a VN antibody response before challenge, however the VN antibody response is faster and stronger than unvaccinated pigs post challenge (Misinzo et al., 2006; Scortti et al., 2007; Zuckermann et al., 2007). However, with the optimized inactivation protocol (Delrue et al., 2009) and adjuvant, it was possible to induce a VN antibody response with an experimental BEI-inactivated Marc-145 grown LV in an oil-in-water adjuvant vaccine (Vanhee et al., 2009). In this thesis, vaccination with BEI-inactivated, Marc-145 grown 07V063 induced a VN antibody response before challenge, while vaccination with BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown 07V063 only primed a VN antibody response after challenge. Vaccination with BEI-inactivated, Marc-145 grown 07V063 with a vaccine dose of 10^9 $TCID_{50}$ resulted in a reduction of viremia upon challenge, while a vaccine dose of 10^8 $TCID_{50}$ did not, indicating that the vaccine dose of 10^8 $TCID_{50}$ was too low. Both vaccinations based on BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown 07V063 with a vaccine dose of 10^9 $TCID_{50}$ and 10^8 $TCID_{50}$ were able to reduce viremia. If the vaccine virus was produced in $PK15^{Sn\text{-}CD163}$ cells, a vaccine dose of 10^8 $TCID_{50}$ was sufficient to obtain a reduction in viremia. To obtain a similar reduction of viremia using Marc-145 grown vaccine virus, a vaccine dose of 10^9 $TCID_{50}$ was necessary. Viremia can be suppressed by commercial MLV vaccines (Zuckermann et al., 2007), but mostly only a partial reduction can be achieved (Diaz et al., 2006; Prieto et al., 2008). Also for commercial and experimental KV vaccines only a partial reduction of viremia can be achieved (Misinzo et al., 2006; Vanhee et al., 2009; Zuckermann et al., 2007). It is shown that if VN antibodies are present at time of challenge, infection can be prevented (Osorio et al., 2002). However, KV vaccines cannot induce a VN antibody response that can sustain until challenge, which can explain the only partial reduction of viremia. Interestingly, three weeks post challenge some vaccinated pigs became virus-positive again. This can be due to the presence of an escape mutant leading to evasion of the immune system and viral persistence as described for Hepatitis C virus (Uebelhoer et al., 2008). Whether this is also the case for PRRSV will be studied in the future. To conclude, PRRSV induces an inefficient humoral and cellular immune response (Meier et al., 2003). A full protective immune response is difficult to induce with MLV as well as with

KV vaccines. In addition, PRRSV shows a wide spread genetic diversity, which makes it complicated to generate a proper PRRSV vaccine.

Conclusion

An ideal PRRSV vaccine should meet a number of requirements. First, it should contain vaccine virus that is related to the circulating virus strain. This requirement is most easily met by generating a KV vaccine. Second, the vaccine virus should be produced on a cell line that avoids mutations in the viral structural proteins. For a KV vaccine, the vaccine virus should be completely inactivated with conservation of the viral structural proteins. For an MLV, the mutation in non-structural proteins should be stable. The vaccine should contain an efficient adjuvant and the minimum effective vaccination dose. The production scheme of the experimental inactivated PRRSV vaccine developed in this thesis is shown in Figure 5.2.

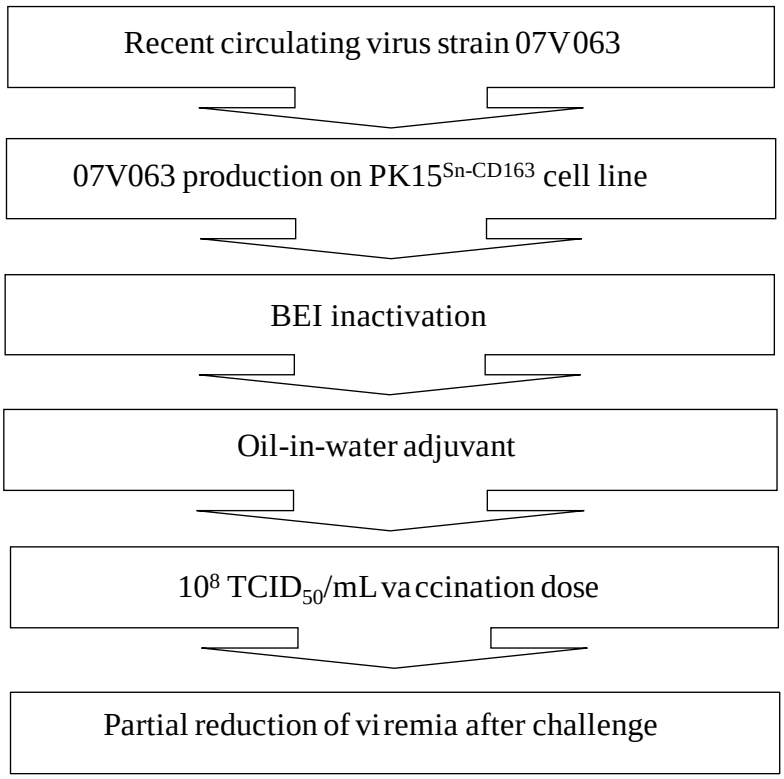

Figure 5.2. Production process of the experimental inactivated PRRSV vaccine developed in this thesis.

The main conclusion on the efficacy of the experimental BEI-inactivated PK15$^{Sn\text{-}CD163}$ grown 07V063 vaccine with oil-in-water adjuvant (an oil-in-water (o/w) diluent that is used in the commercial pseudorabies virus vaccine Suvaxyn Aujeszky (Fort Dodge Animal Health, Kelmis, Belgium)) developed in this thesis is that the vaccination dose can be decreased giving the same protection as compared to Marc-145 grown 07V063. The lowest vaccination

dose tested was 10^8 $TCID_{50}$/mL (titrated on macrophages), but this dose might even be more decreased until 10^7 or 10^6 $TCID_{50}$/mL if the vaccine virus is grown on $PK15^{Sn\text{-}CD163}$ cells. This will be tested in the future. The protection is still only partial, thus it remains a challenge for the future to develop an inactivated PRRSV that can completely block viremia upon infection. This shortcoming might be overcome by using an MLV vaccine for a primo vaccination, since the MLV vaccines on the market can fully protect pigs in a homologous situation, followed by a booster vaccination with a KV vaccine. This will be studied in the future.

The main conclusions that can be drawn from this thesis are:

- A cell line expressing Sn and CD163 ($PK15^{Sn\text{-}CD163}$), two important receptors for PRRSV infection, can be used for vaccine virus production with a yield similar than obtained in Marc-145 cells and with no or minimal mutations in structural viral proteins during adaptation.
- AT-2 cannot inactivate PRRSV and is therefore not useful for KV vaccine development.
- Formaldehyde, glutaraldehyde and pH changes do not preserve the entry-associated domains of PRRSV, which are probably important for the induction of a VN antibody response, and are therefore not useful for the generation of a KV vaccine.
- 37°C, UV irradiation, gamma irradiation and BEI can inactivate PRRSV with preservation of the entry-associated domains and are therefore useful for KV vaccine development.
- Experimental vaccines based on UV- and BEI-inactivated, Marc-145 grown LV strongly prime a VN antibody response and can reduce the duration and severity of viremia upon challenge.
- An experimental vaccine based on BEI-inactivated, $PK15^{Sn\text{-}CD163}$ grown 07V063 can prime a VN antibody response and can reduce the duration and severity of viremia upon challenge.
- If the vaccine virus was produced in $PK15^{Sn\text{-}CD163}$ cells, a vaccine dose of 10^8 $TCID_{50}$ was sufficient to obtain a reduction in viremia. To obtain a similar reduction of viremia using Marc-145 grown vaccine virus, a vaccine dose of 10^9 $TCID_{50}$ was necessary.

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Chapter 6

SUMMARY - SAMENVATTING

SUMMARY

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for reproduction disorders in sows, which leads to tremendous economic losses in the pig industry worldwide. It is also associated with porcine respiratory disease complex (PRDC), which supports massive use of antibiotics. Although several modified live virus (MLV) and killed virus (KV) vaccines are on the market, PRRSV remains difficult to control in the field. The major aim of this thesis was to develop a new experimental inactivated PRRSV vaccine. The different processes to develop a vaccine are virus production, virus inactivation or attenuation and vaccine formulation. In this thesis, the virus production and the virus inactivation of PRRSV were optimized to develop an efficient KV vaccine.

The first section of chapter 1 gives an introduction on PRRSV, more particular its history, classification, virus structure, genomic organization, viral proteins, replication cycle, clinical signs, pathogenesis, innate and adaptive immune response to PRRSV and the available vaccines. A second section of chapter 1 gives an overview of different viral inactivation procedures, such as cross-linkers (formaldehyde, glutaraldehyde and AT-2), denaturing agents (pH and temperature), irradiation (ultraviolet (UV) and gamma irradiation) and alkylating agents (binary ethyleneimine (BEI)), and their effect on viruses.

In chapter 2, the aims of the thesis are formulated.

In chapter 3, different inactivation procedures for PRRSV are screened for their usefulness for the development of an inactivated PRRSV vaccine. Two major concerns in KV vaccine development are (i) the complete inactivation of the virus (safety) and (ii) the preservation of the antigen structure (immunogenicity). As PRRSV neutralizing antibodies can block the interaction of the virus with the internalization receptor sialoadhesin (Sn), the viral entry-associated domains are suggested to be important for the induction of a virus-neutralizing (VN) antibody response. First, the complete inactivation of the virus after treatment with formaldehyde, glutaraldehyde, AT-2, pH changes, 37°C, UV irradiation, gamma irradiation and BEI was evaluated with a virus titration on macrophages. This study showed that for all inactivation procedures, except for AT-2, an optimal condition can be found where PRRSV is completely inactivated. Then the preservation of the viral entry-associated domains, as they seem important for the induction of a VN antibody response, was investigated by monitoring

PRRSV attachment to and internalization into macrophages before and after inactivation. Procedures which affect proteins by cross-linking, like formaldehyde and glutaraldehyde, or by denaturation, like pH changes, resulted in destruction of viral proteins. Upon inactivation by these procedures, PRRSV was not able to attach to and internalize into macrophages anymore and these procedures are therefore not suitable for the production of an inactivated PRRSV vaccine. Inactivated PRRSV at 37°C could still attach to and internalize into macrophages while completely inactivated, which makes increased temperature a candidate for inactivating PRRSV for vaccine development. Procedures which mainly affect the viral genome, like UV irradiation, gamma irradiation and BEI preserved the viral entry-associated domains, as PRRSV inactivated by these procedures was still able to attach to and internalize into macrophages. However, for gamma irradiation the range between complete inactivation of PRRSV and preservation of the entry-associated domains was too small to be useful as an inactivation procedure to generate a safe KV vaccine. UV and BEI are effective inactivation procedures for the production of an inactivated PRRSV vaccine as they can completely inactivate the virus as well as preserve the viral entry-associated domains. To determine the efficacy of the experimental inactivated PRRSV vaccines based on UV or BEI inactivation, pigs were vaccinated twice (6 and 10 weeks of age) with UV-inactivated Marc-145 grown Lelystad (LV), BEI-inactivated Marc-145 grown LV or a commercial KV. After 8 weeks all pigs were challenged with virulent vaccine virus. Serum was tested for virus-specific and VN antibodies and presence of virus. Vaccination with both UV- or BEI-inactivated virus in combination with IFA strongly induced virus-specific antibodies and resulted in an earlier and strongly elevated VN antibody response compared to control animals after challenge. The commercial KV did not induce virus-specific antibodies, and only slightly elevated the VN antibody response after challenge. A significant reduction in virus titer could be observed in animals that were vaccinated with BEI-inactivated virus, while only a slight reduction of viremia was observed with the commercial KV vaccine and UV-inactivated virus.

In chapter 4.1, a PRRSV susceptible cell line ($PK15^{Sn\text{-}CD163}$) was constructed for the production of vaccine virus. To generate an inactivated PRRSV vaccine, cells that are able to produce vaccine virus are necessary. Macrophages, the natural host cells for PRRSV replication, are not useful for virus production, because of batch variation, risk of contamination and high production costs. The PRRSV susceptible cell line Marc-145 can overcome problems associated with the use of primary macrophages. However, as the entry and possibly also the replication pathway of PRRSV in macrophages is different compared to

Marc-145 cells, adaptation of PRRSV for growth on Marc-145 cells is necessary. This adaptation can cause unwanted mutations in viral non-structural and structural proteins. To avoid mutation after adaptation, cell lines expressing Sn and CD163, two important molecules involved in PRRSV infection of macrophages, were constructed, as described in the first section of chapter 4. CHO and PK15 cells were first stably transfected with Sn and selected for clones expressing Sn with geneticin. Afterwards, CHO^{Sn} and $PK15^{Sn}$ cells were transfected with CD163 and selected for clones expressing both Sn and CD163 with zeocin. After investigation of the most PRRSV-sensitive clone and best cultivation conditions, $CHO^{Sn\text{-}CD163}$ clone IC5 and $PK15^{Sn\text{-}CD163}$ clone IXH7 cultivated for two days at a concentration of 200 000 cells/mL were found to be optimal for virus infection. The two cell lines, $CHO^{Sn\text{-}CD163}$ and $PK15^{Sn\text{-}CD163}$, were both susceptible for PRRSV, while the parental cell lines were not. Virus particles of the European prototype LV and the American prototype VR-2332 attached to and internalized into both cell lines, after which disassembly and infection occurred. Not all tested PRRSV strains were able to replicate several passages in $CHO^{Sn\text{-}CD163}$ cells. Only the American prototype VR-2332 and one recent Belgian isolate (07V063) replicated well in this cell line, while the European prototype LV and two other recent Belgian isolates (08V204 and 08V194) did not. The $PK15^{Sn\text{-}CD163}$ cell line was able to produce all tested PRRSV strains with a yield similar to that obtained in Marc-145 cells. In addition, analysis of the sequence of the structural proteins of original virus and virus grown for 5 passages on $PK15^{Sn\text{-}CD163}$ cells showed either no amino acid (aa) changes (VR-2332 and 07V063), one aa (LV), two aa (08V194) or three aa (08V204) changes. However, none of these changes in virus grown on $PK15^{Sn\text{-}CD163}$ cells were situated in known neutralizing epitopes. The $PK15^{Sn\text{-}CD163}$ cell line was thus useful for vaccine virus production, since the virus yield was similar as obtained in Marc-145 cells and no or minimal mutation occurred after adaptation on the cell line.

In chapter 4.2, the efficacy of BEI-inactivated PRRSV vaccines based on virus grown on Marc-145 cells and on $PK15^{Sn\text{-}CD163}$ cells was compared. A recent PRRSV isolate 07V063 was used as vaccine virus and grown on Marc-145 cells or the PRRSV susceptible $PK15^{Sn\text{-}CD163}$ cell line. Afterwards, the virus was inactivated using an optimized inactivation procedure (BEI-inactivation) and two vaccine doses were tested. Pigs were vaccinated twice (6 and 10 weeks of age) with BEI-inactivated, Marc-145 or $PK15^{Sn\text{-}CD163}$ grown virus at two different vaccine doses (10^9 $TCID_{50}$ or 10^8 $TCID_{50}$ on macrophages). After 8 weeks all pigs were challenged with 10^6 $TCID_{50}$ macrophage grown 07V063. Serum was tested for virus-

specific and VN antibodies and presence of virus. Vaccination with BEI-inactivated, Marc-145 and PK15$^{Sn\text{-}CD163}$ grown virus induced a virus-specific antibody response. Marc-145 grown virus induced a VN antibody response, while PK15$^{Sn\text{-}CD163}$ grown virus only primed a VN antibody response. Vaccination with BEI-inactivated, Marc-145 grown virus with a vaccine dose of 10^9 $TCID_{50}$ resulted in a significant reduction of viremia upon challenge, while BEI-inactivated, Marc-145 grown virus with a vaccine dose of 10^8 $TCID_{50}$ did not, indicating that the vaccine dose of 10^8 $TCID_{50}$ was too low. In fact, both vaccinations based on BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown virus (10^9 $TCID_{50}$ and 10^8 $TCID_{50}$) resulted in reduction of viremia. These results indicate that the vaccine dose of vaccines based on virus produced on PK15$^{Sn\text{-}CD163}$ cells can be decreased to have an efficient reduction of viremia.

In chapter 5, the experimental studies are discussed and the main conclusions that can be drawn from this thesis are:

- AT-2 cannot inactivate PRRSV and is therefore not useful for KV vaccine development.
- Formaldehyde, glutaraldehyde and pH changes do not preserve the entry-associated domains of PRRSV, which are probably important for the induction of a VN antibody response, and are therefore not useful for the generation of a KV vaccine.
- 37°C, UV irradiation, gamma irradiation and BEI can inactivate PRRSV with preservation of the entry-associated domains and are therefore useful for KV vaccine development.
- Experimental vaccines based on UV- and BEI-inactivated, Marc-145 grown LV strongly prime a VN antibody response and can reduce the duration and severity of viremia.
- A cell line expressing Sn and CD163 (PK15$^{Sn\text{-}CD163}$), two important receptors for PRRSV infection, can be used for vaccine virus production with a yield similar than obtained in Marc-145 cells and with no or minimal mutations in the viral structural proteins.
- An experimental vaccine based on BEI-inactivated, PK15$^{Sn\text{-}CD163}$ grown 07V063 can prime a VN antibody response and can reduce the duration and severity of viremia.
- If the vaccine virus was produced on PK15$^{Sn\text{-}CD163}$ cells, a vaccine dose of 10^8 $TCID_{50}$ was sufficient to obtain a reduction in viremia. To obtain a similar reduction of viremia using Marc-145 grown vaccine virus, a vaccine dose of 10^9 $TCID_{50}$ was necessary.

SAMENVATTING

Porcien reproductief en respiratoir syndroom virus (PRRSV) is verantwoordelijk voor reproductiestoornissen bij zeugen, die wereldwijd tot hoge economische verliezen in de varkensindustrie leidt. Het is ook geassocieerd met het porciene respiratoir ziekte complex, wat het overmatige gebruik van antibiotica in de hand werkt. Hoewel er verschillende gemodificeerd levend virus (MLV) en geïnactiveerd virus (KV) vaccins op de markt zijn, blijft PRRSV moeilijk onder te controle te houden in het veld. De voornaamste doelstelling van deze thesis was dan ook het ontwikkelen van een nieuw experimenteel geïnactiveerd PRRSV vaccin. De verschillende processen om een vaccin te ontwikkelen zijn virusproductie, virusinactivatie of attenuatie en vaccinformulatie. In deze thesis, werden de virusproductie en de virusinactivatie van PRRSV onderzocht en geoptimaliseerd om een efficiënt KV vaccin te ontwikkelen.

In het eerste deel van hoofdstuk 1 wordt er een inleiding gegeven over PRRSV, meer specifiek over de PRRSV geschiedenis, classificatie, virusstructuur, genomische organisatie, virale eiwitten, replicatiecyclus, klinische symptomen, pathogenese, aangeboren en adaptieve imuunresponsen gericht tegen PRRSV en de bestaande vaccins. Het tweede deel van hoofdstuk 1 geeft een overzicht van de verschillende inactivatieprocedures, zoals cross-linkers (formaldehyde, glutaaraldehyde en AT-2), denaturerende agentia (pH en temperatuur), straling (ultraviolet (UV)-straling en gammastraling) en een alkylerend agens (binair ethyleneimine (BEI)), en hun effect op virussen.

In hoofdstuk 2 worden de doelstellingen van de thesis geformuleerd.

In hoofdstuk 3 wordt voor verschillende inactivatieprocedures voor PRRSV onderzocht of ze nuttig zijn voor de ontwikkeling van een geïnactiveerd PRRSV vaccin. Er zijn twee belangrijke criteria waaraan een KV vaccin moet voldoen: (i) complete inactivatie van het virus (veiligheid) en (ii) het behoud van de antigene structuur (immunogeniciteit). Aangezien PRRSV-neutraliserende antistoffen de interactie tussen het virus en de internalisatiereceptor Sn kunnen blokkeren, worden de virale entry-geassocieerde domeinen belangrijk geacht voor de inductie van een VN antistofrespons. Eerst werd de complete inactivatie van het virus na behandeling met formaldehyde, glutaaraldehyde, AT-2, pH verandering, 37°C, UV-straling, gammastraling en BEI geëvalueerd door een virustitratie op macrofagen. Deze studie toonde

aan dat voor alle inactivatieprocedures, behalve voor AT-2, een optimale conditie kon gevonden worden waarbij PRRSV volledig was geïnactiveerd. Vervolgens werd, aangezien de virale entry-geassocieerde domeinen belangrijk lijken voor de inductie van een virusneutraliserende (VN) antistofrespons, onderzocht of deze domeinen behouden bleven na inactivatie. Hiervoor werden de binding van PRRSV aan en de opname in macrofagen bekeken vóór en na inactivatie. Procedures die een invloed hebben op de eiwitstructuur door crossslinking, zoals formaldehyde en glutaaraldehyde, of door denaturatie, zoals pH veranderingen, resulteerden in degradatie van de virale eiwitten. PRRSV dat met deze procedures geïnactiveerd werd, was niet meer in staat om te binden aan en opgenomen te worden in macrofagen, waaruit besloten kon worden dat deze procedures niet bruikbaar waren voor de productie van een geïnactiveerd PRRSV vaccin. Geïnactiveerd PRRSV bij 37°C kon nog binden aan en opgenomen worden in macrofagen na complete inactivatie, wat het een kandidaat maakt om PRRSV te inactiveren voor vaccinontwikkeling. Procedures die hoofdzakelijk een effect hebben op het virale genoom, zoals UV-straling, gammastraling en BEI konden eveneens de virale entry-geassocieerde domeinen bewaren, aangezien PRRSV geïnactiveerd door deze procedures nog kon binden aan en opgenomen worden door macrofagen. Voor gammastraling was het verschil tussen de stralingsdosis nodig voor inactivatie en de maximale stralingsdosis waarbij de entry-geassocieerde domeinen nog bewaard bleven te klein om deze inactivatieprocedure te kunnen gebruiken om een veilig KV vaccin te maken. UV en BEI zijn wel bruikbare inactivatieprocedures voor de productie van een geïnactiveerd PRRSV vaccin aangezien deze het virus compleet kunnen inactiveren met behoud van de entry-geassocieerde domeinen. Om de effectiviteit te bepalen van een experimenteel geïnactiveerd PRRSV vaccin gebaseerd op UV of BEI inactivatie, werden biggen twee keer (op 6 en 10 weken leeftijd) gevaccineerd met UV-geïnactiveerd Marc-145 gegroeid Lelystad (LV), BEI-geïnactiveerd Marc-145 gegroeid LV of een commercieel KV vaccin. Na 8 weken werden alle varkens aan een challenge met virulent vaccinvirus onderworpen. Het serum werd getest op virusspecifieke en VN antistoffen en aanwezigheid van virus. Vaccinatie met zowel UV- als BEI-geïnactiveerd Marc-145 gegroeid LV in combinatie met IFA induceerde virusspecifieke antistoffen en resulteerde in een vroegere en hogere opkomst van een VN antistofrespons ten opzichte van controle dieren na challenge. Het commercieel KV vaccin kon geen virusspecifieke antistoffen induceren en kon enkel een verhoogde VN antistofrespons na challenge teweeg brengen. Er werd een significante reductie van de virustiter vastgesteld bij de dieren die gevaccineerd waren met BEI-geïnactiveerd

Marc-145 gegroeid LV, terwijl enkel een kleine reductie van de viremie werd bekomen met het commercieel KV vaccin of UV-geïnactiveerd virus.

In hoofdstuk 4.1, werd een PRRSV-gevoelige cellijn ($PK15^{Sn-CD163}$) geconstrueerd om vaccinvirus te produceren. Om een geïnactiveerd PRRSV vaccin te ontwikkelen, zijn cellen nodig om het vaccinvirus in op te groeien. Macrofagen, de natuurlijk gastheercellen voor PRRSV, zijn niet interessant voor virusproductie wegens batchvariatie, risico op contaminatie en hoge productiekosten. De PRRSV-gevoelige cellijn Marc-145 laat ons toe om deze problemen die geassocieerd zijn met het gebruik van macrofagen te omzeilen. Aangezien het opnamemechanisme en vermoedelijk ook de replicatie van PRRSV in macrofagen anders is dan in Marc-145 cellen, is aanpassing van PRRSV voor de groei op Marc-145 cellen noodzakelijk. Deze aanpassing kan ongewenste mutaties in virale non-structurele en structurele proteïnen veroorzaken. Om mutatie na adaptatie aan een cellijn te voorkomen werden er cellijnen die Sn en CD163, twee belangrijke receptoren die betrokken zijn bij PRRSV infectie van macrofagen, tot expressie brengen, geconstrueerd in het eerste deel van hoofdstuk 4. CHO en PK15 cellen werden eerst stabiel getransfecteerd met Sn en kloons die Sn tot expressie brachten werden vervolgens geselecteerd met geneticine. Nadien werden CHO^{Sn} en $PK15^{Sn}$ cellen getransfecteerd met CD163 en kloons die Sn en CD163 tot expressie brachten werden daarna geselecteerd met zeocine. Na optimalisatie van de cultivatiecondities, werden $CHO^{Sn-CD163}$ kloon IC5 en $PK15^{Sn-CD163}$ kloon IXH7 na 2 dagen cultivatie aan een concentratie van 200 000 cellen/mL optimaal bevonden voor virusinfectie. De twee cellijnen, $CHO^{Sn-CD163}$ en $PK15^{Sn-CD163}$, waren beiden gevoelig voor PRRSV. Viruspartikels van het Europese prototype LV en het Amerikaans prototype VR-2332 konden binden aan en opgenomen worden door beide cellijnen, waarna ontmanteling en infectie mogelijk waren. Niet alle geteste PRRSV stammen konden echter groeien in $CHO^{Sn-CD163}$ cellen na verschillende passages. Enkel het Amerikaans prototype VR-2332 en één recent Belgisch isolaat (07V063) konden repliceren in deze cellijn, terwijl het Europese prototype LV en twee andere recente Belgische isolaten (08V204 en 08V194) dit niet konden. In de $PK15^{Sn-CD163}$ cellijn konden wel alle geteste PRRSV stammen geproduceerd worden met een opbrengst die gelijkaardig was dan dat in Marc-145 cellen. Bovendien bleek na een vergelijkende analyse van de sequenties van de structurele eiwitten van origineel virus en virus gegroeid op $PK15^{Sn-CD163}$ cellen na vijf passages, dat er geen aminozuur veranderingen optraden (VR-2332 en 07V063) of slechts één aminozuur (LV), twee aminozuren (08V194) of drie aminozuren (08V204) veranderden. Geen enkele van de aminozuren die veranderden bij het virus

gegroeid op PK15$^{Sn\text{-}CD163}$ cellen waren gelegen in een gekend neutraliserend epitoop. De PK15$^{Sn\text{-}CD163}$ cellijn was dus nuttig voor vaccinvirusproductie, aangezien de virusopbrengst gelijkaardig was als bekomen in Marc-145 cellen en er geen of minimale mutaties voorkwamen na adaptatie aan de cellijn.

In hoofdstuk 4.2, werd de efficiëntie van BEI-geïnactiveerde PRRSV vaccins gebaseerd op virus gegroeid op Marc-145 cellen en op PK15$^{Sn\text{-}CD163}$ cellen vergeleken. Een recent PRRSV isolaat 07V063 werd gebruikt als vaccinstam en dit werd gegroeid op Marc-145 cellen of op de PRRSV-gevoelige PK15$^{Sn\text{-}CD163}$ cellijn. Daarna werd het vaccinvirus geïnactiveerd volgens een geoptimaliseerde inactivatieprocedure (BEI inactivatie), werd een adjuvans toegevoegd en werden twee vaccindosissen getest. Biggen werden tweemaal gevaccineerd (op 6 en 10 weken leeftijd) met BEI-geïnactiveerd, Marc-145 of PK15$^{Sn\text{-}CD163}$ gegroeid virus met twee verschillende vaccindosissen (10^9 $TCID_{50}$ of 10^8 $TCID_{50}$ op macrofagen). Na 8 weken werden alle dieren onderworpen aan een challenge met 10^6 $TCID_{50}$ macrofaag gegroeid 07V063. Serum werd getest op virusspecifieke en VN antistoffen en aanwezigheid van virus. Vaccinatie met BEI-geïnactiveerd, Marc-145 en PK15$^{Sn\text{-}CD163}$ gegroeid virus induceerde een virusspecifieke antistofrespons. Hoewel Marc-145 gegroeid virus een VN antistofrespons induceerde, kon PK15$^{Sn\text{-}CD163}$ gegroeid virus enkel een VN antistofrespons primen. Vaccinatie met BEI-geïnactiveerd, Marc-145 gegroeid virus met een vaccindosis van 10^9 $TCID_{50}$ kon de viremie reduceren na challenge, terwijl een vaccindosis van 10^8 $TCID_{50}$ dit niet kon, wat aangeeft dat deze dosis te laag was. In tegenstelling hiermee, konden beide vaccins gebaseerd op BEI-geïnactiveerde, PK15$^{Sn\text{-}CD163}$ gegroeid virus met een vaccindosis van 10^9 $TCID_{50}$ en 10^8 $TCID_{50}$ de viremie reduceren. Deze resultaten tonen aan dat de vaccindosis voor de vaccins gebaseerd op virus geproduceerd op de PK15$^{Sn\text{-}CD163}$ cellen verlaagd konden worden om met eenzelfde efficiëntie de viremie te kunnen reduceren.

In hoofdstuk 5, werden de experimentele studies bediscussieerd en de belangrijkste conclusies die getrokken kunnen worden uit deze thesis zijn:

- AT-2 kan PRRSV niet inactiveren en is daarom niet geschikt voor KV vaccinontwikkeling.
- Formaldehyde, glutaaraldehyde en pH veranderingen kunnen de entry-geassocieerde domeinen van PRRSV, die waarschijnlijk belangrijk zijn voor de inductie van een VN antistofrespons, niet behouden en zijn daarom niet geschikt voor de ontwikkeling van een KV vaccin.

- 37°C, UV-straling, gammastraling en BEI kunnen PRRSV inactiveren met behoud van de entry-geassocieerde domeinen en zijn daarom geschikt voor KV vaccinontwikkeling.
- Experimentele vaccins gebaseerd op UV- en BEI-geïnactiveerd, Marc-145 gegroeid LV kunnen een VN antistofrespons primen en kunnen de duur en de ernst van de viremie reduceren.
- Een cellijn die Sn en CD163, twee belangrijke receptoren voor PRRSV infectie, tot expressie brengt ($PK15^{Sn\text{-}CD163}$), kan gebruikt worden om vaccinvirus te produceren met een opbrengst die gelijkaardig is dan op Marc-145 cellen en met geen of minimale mutaties in de virale structurele eiwitten.
- Een experimenteel vaccin gebaseerd op BEI-geïnactiveerd, $PK15^{Sn\text{-}CD163}$ gegroeid 07V063 kan een VN antistofrespons primen en kan de duur en de ernst van de viremie reduceren.
- Als het vaccinvirus geproduceerd werd op $PK15^{Sn\text{-}CD163}$ cellen was een vaccindosis van 10^8 $TCID_{50}$ reeds voldoende om een reductie van de viremie te krijgen. Om een gelijkaardige reductie van de viremie te krijgen met Marc-145 gegroeid vaccin virus was een vaccindosis van 10^9 $TCID_{50}$ nodig.

CURRICULUM VITAE

Personalia

Iris Delrue werd geboren in Brugge op 22 februari 1982. In 2000 behaalde zij het getuigschrift van het hoger secondair onderwijs aan het Sint-Andreaslyceum te Brugge in de richting Grieks-Wiskunde. Aan de Universiteit Gent volgde ze de kandituuropleiding in de Scheikunde en de licentiaatopleiding in de Biotechnologie. In 2004 studeerde ze af als licentiaat in de Biotechnologie. Daarna studeerde ze verder aan de Universiteit Gent en behaalde in 2005 het diploma van Master in Advanced Studies in Molecular Biotechnology: option Molecular Medical Biotechnology met onderscheiding. Datzelfde jaar begon ze in het laboratorium voor Virologie, vakgroep Virologie, Parasitologie en Immunologie van de Faculteit Diergeneeskunde, Universiteit Gent aan haar doctoraatsonderzoek. Ze werd gesteund door het IWT landbouwkundig onderzoeksproject “Ontwikkeling van een geïnactiveerd porcien reproductief en respiratoir syndroom virus vaccin ter voorkoming van virusgeïnduceerde reproductiestoornissen”.

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Patenten

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Iris