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Cell-Mediated Immunity in Porcine Reproductive and Respiratory Syndrome Virus

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Porcine reproductive and respiratory syndrome virus (PRRSV) is a significant swine pathogen which exhibits considerable sequence diversity. In an attempt to identify highly conserved T-cell epitopes contained in proteins of this virus, we examined heptadecamer peptides spanning the sequence of the PRRSV nonstructural proteins 9, 10 and 11, all of them are highly conserved, for their ability to elicit a recall proliferative and interferon-gamma response in peripheral blood mononuclear cells obtained from pigs immunized against the type-II PRRSV strain FL-12. These studies led to the identification of seven peptides, two from each NSP 9 and NSP 10 and, three from NSP 11 that appear to contain T-cell epitopes. Comparison of the amino acid sequence of these seven peptide sequences to the analogous sequences from a diverse sample of type-II PRRSV strains indicated that these sequences are highly conserved and thus contain highly conserved T-cell epitopes. The identified epitopes may be important in the formulation of immunogens to provide broad cross-protection against diverse PRRSV strains.

Keywords: Porcine Reproductive and Respiratory Syndrome Virus; Nonstructural proteins; PRRSV Vaccines; T-cell epitopes

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Table of Contents

List of Tables:-

List of Figures:-

Abbreviations

PRRS(V)- Porcine Reproductive and Respiratory Syndrome (Virus)

NAbs- Neutralizing Antibodies

MAbs- Monoclonal Antibodies

CMI- Cell-mediated Immunity

CTL- Cytotoxic T Lymphocyte

MHC- Major Histocompatibility Complex

DCs- Dendritic Cells

PBMCs- Peripheral Blood Mononuclear Cells

IFN-α/β/γ- Interferon-alpha/beta/gamma

NSPs- Nonstructural Proteins

d.p.i- Days Post Infection

PI- Post Infection

CPM- Counts Per Minute

ELISpot- ELISA-Linked Immunosorbent Spot Assay

SFC- Spot Forming Cells

Max.resp- Maximum Response

Tot.resp- Total Response

Avg. resp- Average Response

No. Resp. pigs- Number of Responsive pigs

Avg Resp pigs- Average Responsive pigs

Mean±Std.D- Mean ± Standard Deviation

HLA-Human Leukocyte Antigen

Chapter 1: General Introduction

Objective

The identification of T-cell epitopes in PRRSV is sparse and has been limited to structural proteins. Two distinct regions on the GP5 amino acid sequence from PRRSV genotypes I and II were found to contain T-cell epitopes based on their ability to stimulate IFN-γ secreting cells (Diaz et al., 2009). In addition, one report has recently reported the identification of four T-cell epitopes located on the membrane (M) protein of PRRSV (Wang et al., 2011). Taken together, these reports illustrate an invigorated interest in achieving a more detailed picture of the cell mediated protective immunity against PRRSV. Several examples exist, in the case of other highly diverse and variable RNA viruses such as hepatitis C virus (HCV) (Martin et al., 2004) and HIV (Gruters et al., 2002), indicating that highly conserved, T-cell epitopes are responsible for a broad protection based on CMI. Such conserved regions are often detected in nonstructural proteins (NSPs) of these RNA viruses that are synthesized early during the life cycle of the virus. These NSPs typically constitute structurally constrained, conserved proteins involved in replication of the virus. If animals are preferentially immunized against these conserved epitopes of NSPs, it is possible that such immunization could result in a highly "pan-strain specific" protective immunity. This is the principle applied in the development of several multi-epitope vaccines recently reported for "hard-to-immunize" RNA viruses such as HCV (Martin et al., 2004) or HIV (Gruters et al., 2002; Yang et al., 2002) and also against chronic hepatitis B virus infection (Depla et al., 2008).

Accordingly, we selected a subset of PRRSV NSPs based on their functional role in viral replication and virulence. The ORF1 (b) region of the PRRSV genome contains the NSPs 9, 10, 11 and 12 genes. The NSP 9 is the viral RNA-dependent RNA polymerase (RdRp) required for genome replication and transcription and the NSP 10 encodes for helicase function (Fang and Snijder, 2010). The main criteria used to select NSP 9, NSP 10 and NSP 11 for this study was their highly conserved nature which is consistent throughout the North American PRRSV strains, thus constituting good candidates for the identification of T-cell epitope that could provide target antigens capable of eliciting cross-protective immunity against PRRSV. To achieve this goal, overlapping heptadecapeptides spanning the entire length of the PRRSV NSP 9, NSP 10 and NSP 11 were screened respectively using T-cell proliferation and IFN-γ ELISpot assays. Herein we report the identification of T-cell epitopes mapping to these PRRSV NSPs. Two distinct regions each in NSP 9 and NSP 10 and, three distinct regions in NSP 11 were identified as putative T-cell epitopes. As expected, these epitopes were found to be highly conserved among thirty-four North American PRRSV type-II sequences. Moreover, sixty-five additional sequences analyzed to detect the conservation of those T-cell epitopes also proved positive with a high percentage of conservation throughout the North American PRRSV strain.

Chapter 2: Literature Review

Porcine Reproductive and Respiratory Syndrome Disease

Porcine reproductive and respiratory syndrome (PPRS) leads to respiratory disorder in young pigs. It also causes severe reproductive failure and late abortions in sows. The disease was first reported in 1987 in North America, and the causative agent was isolated and characterized for the first time in Europe in 1991(Wensvoort et al., 1991) and one year later in the United States of America (Collins et al., 1992). PRRS has now emerged in the swine industries worldwide and has caused significant economic loss in the pork industry worldwide (Neumann et al., 2005). The causative agent PRRS virus (PRRSV) is a member of the family Arteriviridae which also comprises of other viruses such as Equine Arteritis Virus (EAV), mouse lactate dehydrogenase elevating virus (LDV), and Simian Hemorrhagic Fever Virus (SHFV) (Cavanagh, 1997).

PRRSV is also known as “Blue-Ear Pig” disease which is primarily transmitted via aerosols. It affects mostly young sows and boars (Rossow, 1998). The important factors responsible for the PRRSV infection in the matured pigs rely on the type of PRRS viral strain, gestation period, breed, gender and immune status of the PRRSV infected sows. PRRSV infected pregnant sows show clinical manifestations which include fever, anorexia, lymphadenopathy, labored respiration, gross and microscopic lesions in the lung, and reproductive failure characterized by delivery of weak/stillborn piglets autolysed fetuses (Christianson et al., 1992; Hopper et al., 1992; Rossow et al., 1994; Terpstra et al., 1991). PRRSV infection in neonatal pigs develops variable clinical signs characterized by severe dyspnea and tachypnea (Cooper et al., 1995; Hopper et al., 1992;

Rossow et al., 1994; Rossow et al., 1995; Wensvoort et al., 1991). Death rate of the PRRSV infected neonatal pigs exceeds up to 100% (Rossow, 1998).

One of the important features of PRRSV infection is prolonged viremia in the infected pigs and this may result in continuous shedding of virus for longer periods without entering a latent stage. Tonsils, lungs and lymphoid organs are the major source of viral RNA during the persistence of PRRSV infection in the infected pigs (Lamontagne et al., 2003). Also, PRRSV RNA has been detected by RT-PCR in the semen of boars evidenced *in vivo* at 92 days post infection (d.p.i) (Christopherhennings et al., 1995). In postnatally infected pigs persistence of PRRSV has been reported for up to 150 days, while naturally infected pigs contained virus even up to 210 days(Cho and Dee, 2006).

Porcine Reproductive and Respiratory Syndrome Virus

PRRSV is a small, enveloped and cytolytic virus with a size of 50-65 nm. This positive-sense single stranded RNA virus carries a methylated cap at the 5' end and a polyadenylated tail at the 3' end of its genome. PRRSV belongs to order Nidovirales, family Arteriviridae, genus Arterivirus and its genomic length is about 15.4 kilobase (Cavanagh, 1997; Conzelmann et al., 1993; Wu et al., 2001). It contains 9 open reading frames (ORFs) among which ORF1a and 1b comprise about 80% of the genome and encode for two polyproteins which, after translation, are proteolytically cleaved into 14 nonstructural proteins (NSPs), e.g., NSP1α, NSP1β and NSP2 to NSP12. Both NSP 9, which encodes the viral RNA-dependent RNA polymerase (RdRp) and NSP 10, which encodes a helicase, are responsible for the viral genome replication and transcription. The RdRp domain is found in the C-terminal portion of replicase subunit, NSP 9, which

contains an additional upstream domain of unknown function. PRRSV RNA helicases are a diverse class of enzymes that uses ATP hydrolysis to unwind RNA duplexes in a 5'-to-3' direction *in vitro*. Like RdRp domain, helicase is part of a larger replicase subunit, NSP 10, which also contains an N-terminal predicted zinc-binding domain comprising of highly conserved 13 Cys and His residues. This particular domain can prove critical for the *in vitro* ATPase and helicase activities of the protein. In addition to the core viral enzymes (NSP 9 and NSP 10), the Nidovirus endoribonuclease (NendoU) domain has been identified in NSP 11 and is N-terminally fused to another domain of unknown function. It has been evidenced through reverse genetics that this particular domain of NSP 11 plays an important role in the replicative cycle of all Arteriviruses (Bautista et al., 2002; Fang and Snijder, 2010). Nedialkova et al has shown that recombinant PRRSV NSP 11 exhibited broad substrate specificity *in vitro* (Fang and Snijder, 2010; Nedialkova et al., 2009). It has also been demonstrated that the NendoU knockout mutants remained capable of RNA synthesis that helped in the prediction that this enzyme may not function in a Nidovirus-specific step of viral synthesis but rather targets unknown cellular substrates(Fang and Snijder, 2010). ORFs 2a, 2b and 3-7 located at the 3' end of the viral genome undergo posttranslational cleavage to produce PRRSV structural proteins (Dea et al., 1996; Meulenberg et al., 1995; Wu et al., 2001). The structural proteins are expressed from the 3' end nested set of sub-genomic m RNA Abs sharing the common leader sequence at the 5' terminus (Meulenberg, 2000; Snijder and Meulenberg, 1998). Glycoprotein 5 (GP5), membrane (M) and nucleoprotein (N) encoded by ORFs 5-7, respectively, are known to be the major components viral particles (Dea et al., 2000). The N protein interacts with viral genome to form the nucleocapsid. M

interacts with GP5 to form a heterodimer which is essential for viral assembly since viral particles are not released in the absence of either GP5 or M protein (Mardassi et al., 1996; Wissink et al., 2005). The minor proteins in viral particles include ORF 2a, 3 and 4 and they encode for GP2a, GP3 and GP4, respectively. These proteins interact with each other and are assembled into the virion as a multiplex complex which is also involved in viral infectivity (Wissink et al., 2005). ORF2b, otherwise called as envelope protein (E), is embedded within the ORF2a (Wu et al., 2001).

In spite of the route of entry, productive infection occurs predominantly in alveolar macrophages of the lung (Murtaugh et al., 2002). *In vitro* studies with the virus infection in these cells have shown their ability to grow in primary cultures of alveolar lung macrophages. PRRSV has a strong restricted cell tropism for certain subpopulations of swine monocyte or, macrophage lineage, notably pulmonary intravascular macrophages, subsets of macrophages in lymph nodes and spleen, and intravascular macrophages of the placenta and umbilical cord (Duan et al., 1997; Lawson et al., 1997). In experimentally infected boars, PRRSV can be detected by PCR in semen samples at 92 d.p.i (Christopher-Hennings et al., 1995; Christopherhennings et al., 1995). Experimental studies have shown that subclinical PRRSV infection can persist in the animals. Thus, persistent infection of PRRSV plays a major role in PRRSV survival and transmission, and will likely pose an obstruction in the PRRS control programs (Bilodeau et al., 1994; Wills et al., 1997).

PRRSV grows well in the African monkey kidney cell MA-104 and its derivatives MARC-145, CL-2621 and CRL11171 (Benfield et al., 1992; Collins et al., 1992; Kim et al., 1993; Meng et al., 1994; Meng et al., 1996). They can also be grown in porcine

alveolar macrophages (PAM) cultures (Wensvoort, 1993; Wensvoort et al., 1991). Swine testis (ST) cells were also reported to support PRRSV replication (Plana et al., 1992). Receptor-mediated endocytosis helps the PRRSV to enter into the host cells (Kreutz and Ackermann, 1996; Nauwynck et al., 1999). Some strains of PRRSV do not propagate in all the cell lines showing variations in their susceptibilities as a result, this indicates the existence of PRRSV variants. Thus, both PAM and other cell lines should be used when attempting virus isolation from clinical samples. The PRRSV virion after attachment with the host cell receptors enters into the cells in a clathrin-dependent manner. The viral genomic RNA transfected in the BHK-21 cells lead to the production of infectious virus. Interestingly, cell receptors play a major role as a determinant in PRRSV cell-tropism. (Meulenberg et al., 1998; Nielsen et al., 2003).

Two independent PRRSV receptors heparin sulfate and porcine sialoadhesin (PoSn) have been identified in PAM. On the alveolar macrophages, the viral protein heterodimer GP5-M interacts with the porcine cell surface receptor porcine sialoadhesin. However, this interaction prevents virus entry into the cells irrespective of the binding of viral particles to the cell surface receptors (Delputte et al., 2005; Delputte et al., 2002). In the meantime, sialoadhesin is responsible for both viral attachment and internalization. During uncoating of the virus, it functions together with CD163 (Delputte et al., 2005; Duan et al., 1998; Van Gorp et al., 2008; Vanderheijden et al., 2003). Minimal levels of PoSn expression by the non-activated monocytes may play important role in susceptibility to PRRSV infection (Kimman et al., 2009).

Genetic and Antigenic diversity among various PRRSV strains

PRRSV has been clinically, genetically and antigenically divided into two types which include European (EU-type-I) and North American (NA-type-II) (Mardassi et al., 1994; Meng, 2000; Meng et al., 1994; Meng et al., 1995b; Morozov et al., 1995; Murtaugh et al., 1995). Both of them cause very similar clinical signs in swine irrespective of their genetic and serological differences.

Genetic diversity

PRRSV is genetically heterogeneous by nature (Meng, 2000). The two genotypes resemble 60% to each other based on their overall nucleotide homology although their biological characteristics are very similar (Kim and Yoon, 2008). The relative nucleotide sequence identity between the US isolates and Lelystad is 45.7% in ORF1b, 65-67% in ORF2, 61-64% in ORF3, 63-66% in ORF4, and 61-63% in ORF5 (Meng et al., 1995a; Meng et al., 1995b). ORFs 6 and 7 genes are relatively conserved among the US isolates or among the EU isolates, but extensive genetic variation was observed in the ORFs 6 and 7 genes between EU and US isolates (Meng et al., 1995a). It has been demonstrated by certain experimental analysis of the nucleotide sequence of ORFs 2-7 of 10 US PRRSV isolates that the genetic distance ranges from 2.5-7.9% and is about 35% between LV and the US isolates (Kapur et al., 1996). GP5, the major envelope protein is highly variable and is only 50-55% identical between the two genotypes (Mardassi et al., 1995; Meng et al., 1995b; Nelsen et al., 1999). This variability is caused due to greater induction of neutralizing antibodies (NAbs) which causes exposure to selective antibody pressure. The ORF1 genomic sequence also differs extensively between the US and the

EU strains by 55% nucleotide sequence identity (Allende et al., 1999; Nelsen et al., 1999). ORF1b is more conserved than ORF1a and shares 63% nucleotide sequence identity with that of LV (Meng, 2000). The pathogenic NA PRRSV isolate 16244B has similar sequence homology with the LV with only 63.4% nucleotide identity. ORF1a polyprotein containing the non-structural part has major differences which shared 47% amino acid identity over 2503 residues of the six NSPs encoded. The greatest diversity is found in the NSP2 genome. About 32% amino acid identity is shared with the NSP2 region of LV. It also contains 120 additional amino acids in the central regions. NSPs encoded by the 5'-proximal and central regions of ORF1b have 66-75% amino acid identity. In addition, the ORF1a-1b frame shift region of 16244b has 98% nucleotide identity with LV (Allende et al., 1999; Meng, 2000).

Current studies have shown that EU-PRRSV is also found in other parts of the world such as Canada (Dewey et al., 2000). Meanwhile, type-II PRRSV has also been found in European region via introduction of live vaccine (Nielsen et al., 2001; Nielsen et al., 2002; Storgaard et al., 1999). It has been noticed that EU-like PRRSV isolates have slowly emerged in the US herds (Fang et al., 2007; Ropp et al., 2004). The remarkable feature in EU-PRRSV which differentiates it from the NA-PRRSV is the deletion of 51 nucleotide in its NSP2 region (Fang et al., 2007). PRRSV genetic heterogeneity has increased over time and co-circulation of different variants and their maintenance may contribute to heterogeneity. This can evident from the analysis of the nucleotide sequence similarity that has decreased up to 4.4% range in between the years 1995 and 2000 (Prieto et al., 2009).

The concept of quasispecies variation was demonstrated by sequence analysis of various PRRSV strains which highlighted the strategies of PRRSV infection spread among interspecies (Goldberg et al., 2003). It has also been reported that there are evidence for quasispecies evolution and emergence of a virus subpopulation during utero infection of pigs with a PRRSV isolate (Rowland et al., 1999). The importance of having a quasispecies population during virus infection will affect vaccine efficacy and may lead to vaccine failure (Domingo et al., 1998; Domingo and Holland, 1992; Duarte et al., 1994). Therefore, future investigation in this regard can prove beneficial in the field o PRRSV immunology.

Other important factors leading to the genetic divergence in PRRSV includes the lack of proof-reading activities of RdRp. RNA recombination is also another serious factor leading to the PRRSV polymorphism (Meng, 2000). Recombination of viral particles containing chimeric ORF3 and ORF4 proteins of PRRSV genome were identified during co-infection of two PRRSV isolates in MA-104 cells which showed an estimated frequency of recombination from <2 to 10%. Moreover, sequence analyses of various field isolates of PRRSV have revealed that RNA recombination of PRRSV can also occur in nature(Yuan et al., 1999).

Antigenic Variation

Antigenic variations among various isolates of PRRSV have been well documented (Meng, 2000). It has been reported about the differential activity of the monoclonal antibodies (MAbs) using the two genetic types of PRRSV, EU- and NA- types. Two MAbs VO17 and EP147 to N protein recognized a conserved epitope in US and EU-

PRRSV isolates. The N-protein MAb SDOW17 works as a common MAb to both NA and EU-PRRSV isolates indicating the presence of a common conserved epitope (Nelson et al., 1993). However, four other MAbs of PRRSV N-protein responded to NA-PRRSV, confirming their strain-specificity (Nelson et al., 1993). Six MAbs raised against British isolate of PRRSV recognized EU isolates but did not react with US isolates (Drew et al., 1995). Five anti-GP5 MAbs from a Canadian isolate were unable to react with LV (Pirzadeh and Dea, 1997). The reactivity of MAbs against GP3, GP4 and N proteins with EU- and US-PRRSV isolates revealed antigenic differences both within the isolate as well as between the isolates (Katz et al., 1995; Wieczorek-Krohmer et al., 1996).

The degree of antigenic variation within each PRRSV type is highly considered during the design of future vaccines. A multivalent vaccine comprising of multiple antigenically distinct strains of PRRSV can prove as the most promising candidate for the next generation of vaccines (Meng, 2000).

Immune Response

Development of a weak and delayed protective immunity against PRRSV infection leads to prolonged acute and persistent infection in pigs. The acute post-infection is followed by the presence of clinical symptoms and abundant replication in target cells such as alveolar macrophages. Acute post-infection is characterized by high viral load in tissues and by the presence of cell-free (serum associated) viremia for one month. The persistent phase of PRRSV involves low level of viral replication. 100% viral clearance was seen in at least more than 150 days post-infection. A period of two months post-infection for effective contagion to other animals has been seen early after exposure a vigorous anti-

PRRSV antibody response at 7-9 days post-infection was detected by ELISA. Antibodies with PRRSV-neutralizing activity appear only at later post-infection times (≥ 4 weeks), leading to polygonal B-cell activation. Presence of total antibodies in serum during early post-infection viremic phase of PRRSV infection indicates about the inability of antibodies to play a role in protection against PRRSV infection. Anti-PRRSV antibodies constitute a non-protective but deleterious response. Due to slow, irregular appearance of PRRSV-NA after PRRSV exposure, it was able to prevent the appearance of viremia. Infected pigs develop a T-cell mediated response at 4 weeks PI indicating a CD4 T-cell response. Specific IFN-γ production by T-cells (CTL specific response) showed a delay in the response similar to the one observed with NA. Mostly infected pigs shed viral infection at approximately 3-4 months of post-infection. Therefore, it is difficult to gain a complete solid protection against PRRS infection (Lopez Fuertes et al., 1999; Lopez and Osorio, 2004; Meier et al., 2003). Moreover, very little information is known about the PRRSV structural and nonstructural proteins that can provide protective immunity against PRRSV infection.

Innate immunity

Host innate immunity exhibits a crucial role in the PRRSV immunology. Type-I interferons (e.g., IFN-α/β) display innate protection against PRRSV infection (Seth et al., 2006). Various experimental studies have reported that PRRSV is sensitive to the antiviral effects of type-I IFN (Buddaert et al., 1998; Lee et al., 2004; Miller et al., 2004). *In vitro* studies have shown that upon pretreatment of PAM culture with porcine IFN-α prior to PRRSV infection has resulted in significant reduction in virus titer (Albina et al., 1998; Buddaert et al., 1998; Lee et al., 2004). The sensitivity of PRRSV to IFN effects is

PRRSV isolate-dependent(Lee et al., 2004). Several studies have revealed that IFN-α helps in the clearance of viral activity in infected pigs. PRRSV infection alone is unable to IFN-α. Interestingly, pigs which were infected with Corona virus prior to PRRSV infection, resulted in potential induction of IFN-α, thereby, deliberately reducing viral titers (Buddaert et al., 1998). It has also been evidenced that PRRSV is a poor inducer of type-I IFNs (Albina et al., 1998; Lee et al., 2004; Miller et al., 2004). Moreover, it has been suggested that PRRSV may actively suppress type-I IFN production (Albina et al., 1998). PRRSV infection in pigs rarely elicit IFN-α in their sera and lung secretions (Albina et al., 1998). *In vitro*, PRRSV infection to PAM cells do not show marked induction in the IFN-α production (Albina et al., 1998; Lee et al., 2004). Also, MARC-145 infected with PRRSV has shown alteration in the production of both IFN-α/β m RNA Abs in response to double stranded RNA stimulation (Miller et al., 2004). The viral particles important for the potent elicitation of type-I interferons have not been indentified clearly. Recent works have been published to show that few PRRSV proteins such as NSP1, NSP2, NSP4, NSP 11 and N-protein are involved in IFN-β production (Beura et al.). It has also been proved that PRRSV infection inhibited function of IFN-β promoter stimulator 1 (IPS-1) molecule of the retinoic acid –inducible gene I (RIG-I) or TLR-independent dsRNA signaling pathway which resulted in the inhibition of synthetic dsRNA-induced IFN-β production and IRF3 nuclear translocation (Luo et al., 2008). An intriguing observation is that the production of IFN-γ response in swine serum lasts approximately for 3 weeks (Wesley et al., 2006). Serum levels with increased amount of viral load and inhibition of PRRSV replication by IFN-γ lead to the conclusion that IFN-γ may have a crucial role in the PRRS disease (Bautista and Molitor, 1999; Gaudreault et

al., 2009; Loving et al., 2008). Generally early production of IFN-γ could be due to the activation of natural killer (NK) cells and alveolar macrophages. These pathways have not been identified yet in PRRSV.

Acquired immunity

The delayed immune response developed for protection against viral infection is a debated issue. It takes at least 3 months of time period to develop immunity against natural infection. Moreover, this does not confer a solid protection to prevent re-infection, especially caused by heterologous PRRSV strains (Murtaugh et al., 2002; Zuckermann et al., 2007). Experimental studies have shown that gilts infected with an US-PRRSV type isolate and challenged with EU-PRRSV isotype (Lelystad virus) late in gestation period provided partial protection against the transplacental infection, whereas all the gilts challenged with the homologous virus were completely protected (Lager et al., 1999). These studies provided information about the existence of the partial heterologous protection and also indicate that common epitopes are likely involved in protection against EU- and US-PRRSV strains. As a result, it has become important to understand the acquired immunity in spite of a broad genetic and antigenic variation of circulating strains of PRRSV which can help to induce IFN-γ to provide a protective immunity against PRRSV infection. Moreover, it has been suggested by Diaz et al., that the different capacity of PRRSV strains to induce protective immunity depends on their different capacity to induce a strong cellular, in particular IFN-γ, immune response(Diaz et al., 2006).

Humoral immunity

An important aspect of PRRSV infection is the delayed development of NAbs. PRRSV-specific antibodies begin to appear in the infected pigs as early as 7-10 d.p.i with a low viral titer (Loemba et al., 1996; Plagemann, 2006). The main feature of the onset of humoral immune response consists of early production of low leveled non-NAbs followed by delayed NAbs between 2-4 weeks PI (Labarque et al., 2004; Loemba et al., 1996). It has been demonstrated that there may be a high induction of NAbs and virus *in vivo*, but on experimental basis both NAbs and non-NAbs lead to PRRSV replication in macrophages. As a result, these antibodies can bind to the virus and internalize the viral particles into macrophages (Diaz et al., 2006). *In vitro* studies have demonstrated PAM and MARC-145 cells are prone to secondary PRRSV infection even after the development of early antibodies from the first infection (Nelson et al., 1994; Yoon et al., 1994; Yoon et al., 1995). Antibodies which are passively transferred from pregnant sows to the off springs provide protection against PRRSV infection and help to block transplacental PRRSV infection (Lopez et al., 2007; Osorio et al., 2002). This shows antibodies are important in protecting swine against re-infection with PRRSV. However, addition of NAbs helped in the suppression of viral infectivity in the already infected PAM and MARC-15 cells. This was achieved by blocking viral attachment and internalization (Delputte et al., 2004). Generally, NAb were first detected in serum at about 4 weeks PI (Lopez and Osorio, 2004; Mateu and Diaz, 2008). Thus, inefficiency of NAbs and early antibodies towards developing a solid protection confirmed that antibodies alone cannot protect the pigs against PRRSV infection. It was further confirmed from experimental studies that induction of viral infection and replication is

further supported by the antibody production. NAbs pass from mother to their off springs via colostrums and milk to provide protection against PRRSV infection (Yoon et al., 1996). Importantly, there is a chance of NAbs getting transferred from sows to their offspring maternally through the colostrum and milk. Thus, it provides sterilized immunity in the new-born piglets. Labarque et al., from his findings has suggested one most important role of NAbs in virus clearance related with the clearance of PRRSV from the lungs of infected pigs and the appearance of neutralizing antibodies in sera and bronchoalveolar fluids (Labarque et al., 2004).

PRRSV nucleocapsid protein carries the highest immunogenic properties among all the PRRSV proteins. High levels of anti-N antibodies could be detected at 7 d.p.i, but these do not provide neutralizing property and hence, do not correlate with protection against PRRSV infection. However, these are unable to neutralize the virus. It has been reported that viral epitopes capable of inducing NAbs resides on the GP2a, GP3, GP4, GP5 and M proteins (Meulenberg et al., 1997; Ostrowski et al., 2002; Plagemann et al., 2002; Yang et al., 2000). Among these, NAbs against GP5 of both European and North American PRRSV happen to be most relevant for protection as major neutralization epitope of PRRSV are located in the middle of the GP5 ectodomain (Plagemann et al., 2002). The neutralizing activity of MAbs against GP5 has been known to show stronger activity than that of antibodies to the GP4 (Weiland et al., 1999). GP5-based neutralizing epitope of NA-PRRSV denoted as Epitope B is highly conserved (Ostrowski et al., 2002; Plagemann, 2004). An additional immunodominant non-neutralizing decoy epitope, epitope A, present in the upstream of the epitope B interferes with the immune response to epitope B (Fang et al., 2006). It has been shown that the proximity of epitopes A and B

is a compulsory requirement in delayed NAb response (Fang et al., 2006). The flanking N-linked glycosylation sites on both sides of the epitope B exhibits poor immunogenicity of the epitope B. Hence, it leads to an early and high induction of NAbs response as compared to the wild-type virus (Mateu et al., 2006).

It has also been reported that the M protein contain two neutralizing epitopes. It has been shown that the GP5-M interaction has been used for the construction of a DNA vaccine but NAbs titers induced by this vaccination protocol were low (Jiang et al., 2006). However, GP3 and M protein NAbs remain unidentified yet. Interestingly, the neutralizing ability of GP3-specific antibodies has been mentioned due to its characterization as a NSP in some NA-PRRSV isolates as well as a structural protein in some EU-PRRS isolates (Meulenberg and Petersen-den Besten, 1996). Also, EU-PRRSV contains a neutralizing epitope in its ORF4 region (Vanhee et al.).

Cell-mediated immunity

Cellular mediated immunity (CMI) plays an important role in PRRSV immunology. Previous studies have shown that pigs recovering from experimental PRRSV infection develop strong lymphocyte proliferative and NA responses after four weeks PI (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). T-cell responses were mainly categorized by type-I cytokine expression phenotype with IFN-γ as the major cytokine and, to a lesser extent, IL-2 (Lopez Fuertes et al., 1999). These kinds of cytokine expression are generally identified between 4-12 weeks PI (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). Post-vaccination with US-PRRSV modified live vaccine have led to the virus-specific IFN-γ secreting cells which appeared first in the third week post-vaccination and

increased the number of peripheral blood mononuclear cells (PBMCs) at 48 weeks post-vaccination (Meier et al., 2003). The double positive cells (CD4+CD8+) present in the porcine immune system are responsible for IFN-γ secretion (Meier et al., 2003). An identical immune response with delayed development of PRRSV-specific IFN-γ secreting cells was evident after infection or vaccination with European strains of PRRSV (Diaz et al., 2005; Diaz et al., 2006). However, a live attenuated PRRSV vaccine that induced high IFN-γ secreting cell frequencies protected pigs against viremia. IL-10 levels have also been observed to inversely correlate with the IFN-γ responses. These results include the involvement of a strong T-cell immunity, IFN-γ and IL-10 in the development of immunity against PRRSV(Diaz et al., 2006). This indicates towards the switching over of the immune response from Th1-Th2-mediated immune response. However, GP2(a/b), GP3, GP4, GP5, M and N proteins of PRRSV were examined individually to validate their capability to induce T-cell proliferation. Upon individual expression of these ORFs using vaccinia virus system it was revealed that the M protein was the strongest inducer of proliferation (Bautista et al., 1999; Jiang et al., 2007a; Jiang et al., 2007b; Lopez Fuertes et al., 1999). There is a possibility that PRRSV may interfere with correct antigen presentation and activation of T lymphocytes. PRRSV caused down-regulation of the expression of major histocompatibility complex (MHC)-I in dendritic cells (DCs), in spite of its correlation with the impaired proliferative responses in the mixed leukocyte reaction (Loving et al., 2007). Expression of MHC-I, MHC-II and CD14 was down-regulated in monocyte-derived DCs infected with infectious PRRSV (Wang et al., 2007). Decreased proliferative responses were observed when infected DCs were used with syngeneic or allogeneic lymphocytes. This again suggests that infected DCs present

antigens less efficiently (Wang et al., 2007). By altering the cytokine patterns of macrophages and dendritic cells, as well as by modifying the expression of molecules involved in antigen presentation PRRSV can down-regulate the innate immune response (Mateu and Diaz, 2008). It has been noted that PBMCs isolated from infected pigs upon treatment with CD4 and MHC class-II antibodies tend to reduce around 85% in the T-cell proliferation (Lopez Fuertes et al., 1999). This led to a conclusive remark that CD4+ T-cells are specifically induced during T-cell proliferation. In fact, these infected PBMCs showed greater IL-2 and IFN-γ response in comparison to IL-4 and IL-10. This demonstrates that the CD4+ T-cells predominantly function in CMI response (Lopez Fuertes et al., 1999).

ELISpot technology is a widely used assay to detect the CMI response against PRRSV infection. These can be well predicted by estimating the IFN-γ secreting cells (IFN-γ-SC) in the PBMCs at 14 d.p.i. The frequency of IFN-γ-SC increases to its maximal levels at 28 d.p.i and then slowly declines (Diaz et al., 2005; Diaz et al., 2006; Xiao et al., 2004; Zuckermann et al., 2007). Alternate reports have conveyed that IFN-γ-SC remain undetected until 8-10 weeks PI. But its frequency gradually increases by 48 weeks PI and remains stable until 690 d.p.i (Meier et al., 2003). However, the ability of PRRSV to induce IFN-γ-SC is comparatively low and delayed (Meier et al., 2003).

For most of the proteins T-cell epitopes have not yet been identified at amino acid level. Consequently it is unknown whether conserved T-cell epitopes might provide cross-protection against different PRRSV strains. Determination of T-cell epitopes is an expensive and cumbersome task due to the lack of a systematic approach based on the synthesis and testing of large sets of overlapping peptides. Various strategies have been

used to determine the PRRSV T-cell epitopes only in structural proteins. ELISpot assay has been used recently by Vashisht et al., which helped them to identify two distinct regions (amino acid residues 117-131 and 149-163) on GP5 of the North American genotype of PRRSV that appeared to contain immunodominant T-cell epitopes (Vashisht et al., 2008). Moreover, bioinformatics approach along with the ELISpot assay has helped Diaz et al., group to detect two distinct regions on the GP5 amino acid sequence from PRRSV genotypes I and II (Diaz et al., 2009). In addition, one report has recently reported the identification of four T-cell epitopes located on the membrane (M) protein of PRRSV(Wang et al., 2011). Taken together, these reports illustrate an invigorated interest in achieving a more detailed picture of the cell mediated protective immunity against PRRSV. Several examples exist, in the case of other highly diverse and variable RNA viruses such as hepatitis C virus (HCV) (Martin et al., 2004) and HIV (Gruters et al., 2002), indicating that highly conserved, T-cell epitopes are responsible for a broad protection based on CMI. Such conserved regions are often present in the NSPs of these RNA viruses that are synthesized early during the life cycle of the virus. These NSPs typically constitute structurally constrained, conserved proteins involved in replication of the virus. Thus, it has become essential to determine T-cell epitopes in those proteins and vaccinate the PRRSV infected pigs with those conserved epitopes to develop "pan-strain specific" protective immunity.

Vaccine Development against PRRS disease

Multiple vaccines against PRRSV infection are available commercially available. These include inactivated-vaccines, modified-live vaccines, DNA vaccines and recombinant DNA vector vaccines (Kimman et al., 2009). Current vaccines against PRRSV have

several drawbacks. Modified live vaccines protect against homologous isolates but generally have a limited effect against, challenge with heterologous viruses. Live PRRSV vaccines provide partial protection against clinical disease but do not prevent infection. Also, it can revert to virulence. Attenuated vaccines work similar to natural PRRSV infection with low NA production. Killed vaccines have proved less effective in prevention of both infection and disease. NA epitopes have also been developed to generate new vaccines for protective response. At the same time, onset of IFN-γ inducing CTL-response as well as CD4 T helper immune response as delayed immune response may provide protective immunity. Both NA and virus-specific IFN-γ producing cells have played important role in the designing of vaccines (Lopez and Osorio, 2004; Osorio et al., 2002; Zuckermann et al., 2007). It has been reported that PRRSV-NA helps in the prevention of infection of pregnant sows as well as transplacental infection (Osorio et al., 2002). A killed vaccine inducing NAbs failed to protect pigs against an *in vivo* challenge with the virus (Zuckermann et al., 2007). These pigs displayed a secondary neutralizing antibody response upon challenge inoculation, thus suggesting the inefficiency of recall response to provide full protection.

The inactivated-vaccines contain adjuvant. Killed-vaccine has failed to provide cross-protection against PRRSV infection. This was highly evident from the clinical signs, reproductive failure, constant magnitude of viremia in semen among homologous strains and congenital infection in the off springs. Also it remained unaffected in the sows and boars (Scortti et al., 2007). Administration of killed-vaccine in boars did not change and magnitude of viremia and shedding of virus in semen even against homologous challenge (Nielsen et al., 1997).

Modified-live vaccines (MLVs) have shown greater effectiveness in reducing disease probability, and also during viremia and virus shedding. Protection by modified live vaccines has shown solid protection against clinical disease induced by homologous infection while the level of protection induced by MLVs against heterologous strains is variable and substandard (Meng, 2000). However, MLVs were incapable to provide long term protection against PRRSV infection (Christopher-Hennings et al., 1997; Diaz et al., 2006; Labarque et al., 2004; Martelli et al., 2007; Okuda et al., 2008; Prieto et al., 2008; Zuckermann et al., 2007). Simultaneously, MLVs prepared by targeting GP5 provided complete protection against PRRSV (Labarque et al., 2004). It has been examined with the so-called therapeutic vaccine intervention that it was unable to eliminate wild-type PRRSV, but it significantly reduced the number of pigs persistently infected with a homologous strain, but not pigs persistently infected with a heterologous strain (Cano et al., 2007a; Cano et al., 2007b). Live-attenuated PRRSV vaccine (Ingelvac PRRS MLV^{R}) has been examined to check the adjuvant effects of several adjuvants on its protective efficacy against PRRSV infection. Co-administration of IL-12 significantly helps in the induction of the cell-mediated immune response to MLV vaccine as compared to MLV alone (Charerntantanakul et al., 2006).

Various recombinant virus vector systems such as live attenuated vaccine-based Pseudo rabies virus recombinant (strain Bartha)(Qiu et al., 2005), replication-defective adenovirus recombinants(Jiang et al., 2008) and *Mycobacterium tuberculosis* strain BCG(Bastos et al., 2004) has been used against PRRSV infection to induce immunity to PRRSV. All the above data available so far suggest that all structural proteins of the virus are essential for the production of infectious virus. Role of nonstructural proteins (NSPs)

in the field of PRRS immunology has not been implemented yet. Their functional and structural constraints can make many changes in the development of vaccine against PRRSV. As NSPs are highly conserved among the various strains of the homologous as well as heterologous genotypes, they can prove beneficial in cell-mediated immune response. A safe, universal and efficient vaccine can provide a core protection to treat PRRS affected pigs. The first priority depends on the detection of B- and T-cell epitopes involved in the development of protective immunity. Neutralizing epitopes have been established definitively but very little is known about T-cell epitopes, although T-cell responses to individual PRRSV polypeptides have been reported in virus-infected animals. The most important criteria performing this kind of experiment involve the genome conservation of those epitopes in both European and American strains (heterologous protection) as well as among the homologous strain (within American or, European) of PRRSV(Bautista et al., 1999). The next important requirement is the involvement of the components of the virion or viral genome responsible for viral down-regulation or modulation of the swine immune system. The importance of a universal vaccine not only includes immunological properties but, also the characteristics of the strain, relationship between the immunopathological properties and genetic diversity matters a lot (Mateu and Diaz, 2008). The third criteria incorporate the avoidance of the possibilities of reversion of a viral strain in to virulence and also minimal transmission of the vaccine strain between pigs. The obvious way to avoid this kind of possibilities is by using non-replicating vaccines which are again questionable on their ability to induce NAbs and adequate cell-mediated immune responses (Zuckermann et al., 2007). Subunit or vector-based vaccines and inclusion of adjuvant can take active replacement on these

parameters. The last important thing is the development of a differential vaccine. An extensive study of essential and non-essential parts of the viral genome has not been performed. The occurrence of natural variants with deletions in NSP2 indicated the purpose of NSPs as better targets in the designing of these kinds of vaccines (Fang et al., 2004).

Thus, it has become important to detect the T-cell epitopes and prepare a vector-based universal vaccine against PRRSV infection. In addition, it is necessary to construct vaccines that provide protection in both homologous and heterologous strains against PRRSV infection.

Chapter 3: Materials and Methods

General experimental design

Figure 1 illustrates the experimental design used in this study to identify T-cell epitopes in PRRSV nonstructural proteins NSP 9, NSP 10 and NSP 11. To immunize the principal group against PRRSV FL-12 animals were initially infected with a single intramuscular injection of $10^{6.5}$ $TCID_{50}$ of PRRSV FL-12 followed by two additional boosters of the same dose of PRRSV FL-12 given several weeks apart (see below)(Osorio et al., 2002). The first booster was administered emulsified in Freund's complete adjuvant and the second was administered in incomplete Freund adjuvant. The screening tests for NSP 9 and NSP 10 were performed independent of each other at different time points and with different sets of pigs. To screen the NSP 9 peptides for their ability to stimulate a recall T-cell response, a total of sixteen 4-5 weeks old, Landrace x Large White were obtained from a PRRSV-free farm. Eight of those animals were immunized against PRRSV and the other eight were used as non-immune specificity controls. The first booster of the principal group was administered at 21 d.p.i and the second at 96 d.p.i. For NSP 10 screening, we used a total of six mixed-breed animals (same farm of origin, age, and genetic background as previous experiment, 4 PRRSV-immunized principals and 2 uninfected controls). In this case the first booster was administered at 57 d.p.i and the second at 114 d.p.i. For both NSP 9 and NSP 10 experiments, starting at day 7 d.p.i and for the entire length of immunization in each case, blood was collected weekly for the following assays: 1) confirmation of viral infection parameters (viremia, T-cell proliferation and IFN-γ secreting cells by ELISpot) and 2) testing the set of peptide pools as well as to confirm individual peptides. NSP 11

experiments were conducted using cryopreserved PBMCs isolated during NSP 10 animal experiments.

For, NSP 9, NSP 10 and NSP 11 experiments, the animal groups were separately housed in isolated bio-safety level 2 (BL-2) rooms to avoid cross-contamination. All the rooms were well-ventilated and supplied with standard diet ad libitum and proper waste disposal systems to avoid cross-contamination at all times.

Virus strain

To immunize the principal animals we used PRRSV FL-12 strain, which is a highly pathogenic PRRSV type II strain derived from the infectious clone of PRRSV NVSL 97-7895 (Truong et al., 2004) (GenBank accession no. AY545985).

Isolation and cryopreservation of peripheral blood mononuclear cells (PBMCs)

Ten ml of whole blood was collected in a 5mM heparin tube and PBMCs were isolated by density gradient centrifugation using the Lymphocyte Separation Media (Cellgro:Mediatech; cat # 25-072-CV) as previously described were washed using Hyclone RPMI media without 10% fetal bovine serum (FBS, SAFC; cat # 12003C). Then RPMI (10% FBS) was added to the isolated PBMCs obtained and the number of cells were counted using haemocytometer. Purified PBMCs were used for the T-cell proliferation assay. The purified PBMCs were also cryopreserved at a concentration of $3x10^7$ viable cells/ml for ELISpot assay (Kreher et al., 2003). Pigs were bled once every week for testing the set of peptide pools as well as to confirm individual peptides. Cryopreservation of PBMCs was done at different time points including the first and second post booster doses.

Synthesis of PRRSV NSP 9, 10 and 11 synthetic peptides

Three libraries of synthetic heptadeca peptides, with a 9 amino acid overlap, encompassing the entire sequence of NSP 9 (n=79, encompassing 646 aa), NSP 10 (n=54, encompassing 441 aa) and NSP 11 (n=27, encompassing 223 aa) were obtained from NEO Peptide (Massachusetts) (Table 1). These peptides were used at a working concentration of 3mg/ml. Initially, these peptides were used in the form of peptide pools, each pool consisting of four overlapping 17-mer peptides located consecutively in the respective gene sequence (each peptide concentration 20ug/ml). Peptides from responding peptide pools were screened individually for the identification of all individual peptides eliciting immunogenic responses and hence, possessing potential for being T-cell epitopes. Twenty NSP 9, fourteen NSP 10 and seven NSP 11 peptide pools were prepared for the T-cell epitope mapping study (Table 1).

T-cell proliferation Assay

PBMCs were obtained after thorough centrifugation using Lymphocyte Separation Media (Cellgro:Mediatech; cat # 25-072-CV). Cells were counted using a haemocytometer to estimate the number of PBMC used to perform the *in vitro* proliferation assay. Cells at a concentration of 4 x 10^6cells/ml in RPMI medium with 10% FBS were used for the assay. A volume of 100µl cell suspensions was added to 100µl of synthetic peptides at a total concentration of 20µg/ml adjusted in RPMI (10% FBS) and placed in the wells of 96-well U-bottom plate. Plates were incubated for 48 hours at 37°C in 5% CO_2. Twenty microliters of [^{3}H] Thymidine (corresponding to 1µCi per well; M P Bioquote) prepared in cloning medium was added into each well. The plate was incubated for 16 hours at

37°C. Cells were harvested using a PerkinElmer filtermate harvester. [^{3}H] Thymidine incorporation was measured using a scintillation counter (PerkinElmer 1450 LSC). Two rounds of T-cell epitope screening were performed using this assay. The first round of screening was performed using the peptide pools and in the second round the positively responding (proliferating) peptide pools were checked for the T-cell proliferating activity induced by individual peptides. A peptide or pool was considered likely to contain a T-cell epitope on the basis of stimulation index: the frequency of the proliferating cells in response to the peptides which is higher by ≥ 2 folds than the control cells (media only) alone (Vashisht et al., 2008). These experiments were repeated at least three times for all the three NSP 9 and NSP 10, except NSP 11 proteins. Ten microgram per ml of Concanavalin A was used as positive controls to validate the proliferation ability of the cells cultured with peptides in all cases (principals and control animals).

IFN-γ ELISpot Assay

The PRRSV-specific IFN-γ ELISpot assay was previously studied and well established to detect T-cell epitopes (Diaz et al., 2009; Vashisht et al., 2008). Ninety-six well Millipore plates with PVDF membrane were selected to perform this assay (reference). To pre wet the membrane, 15µl 70% ethanol was added to each well and incubated at room temperature for 30 seconds. Primary antibody (porcine IFN-γ P2G10; BD Biosciences Pharmingen, 50µl at 10µg per ml concentration) was added to each well and incubated overnight at 4°C. The plate was washed six times with 0.05% sterile PBS-Tween20 and blocked with 150µl PBS-BSA 1%. It was then incubated at 4°C overnight. The plate was then washed with sterile PBS once and in each well 5 x 10^5 PBMCs were added in media containing peptides selected from T-cell proliferation assay at 37°C overnight. Ten

microgram per ml Concanavalin A and PRRSV FL-12 strain ($10^{6.78}$ $TCID_{50}$ in infected cell homogenate) were used as positive controls to validate the gamma interferon responsiveness of the cells cultured with peptides. Plates were incubated at 37°C overnight. The plate was washed six times with PBS-Tween20 0.05% (sterile). Then, 100µl secondary antibody (biotin P2C11; BD Biosciences Pharmingen) at 2µg per ml concentration was added to each well and incubated at 4°C overnight. The plate was washed six times with sterile PBS-Tween20 (0.05%) and incubated at 4°C for 45 minutes after adding 100µl streptavidin alkaline phosphatase (Southern Biotech; cat. # 7100-04) dissolved in PBS-BSA1% in 1:1000 dilutions. Subsequently, the plate was washed three times with sterile PBS-Tween20 (0.05%) followed by three washes with PBS. 100µl alkaline phosphatase substrate (Vector laboratories; cat. # SK-5300) was added to each well and kept at room temperature for 7-10 minutes. To stop further spot development, the plate was then washed extensively under running water to stop further spot development. The number of spot forming cells (SFCs) was counted using the C.T.L ELISpot machine. These experiments were repeated at least three times for NSP 9, NSP 10 and NSP 11. This assay was performed using both cryopreserved cells and fresh PBMCs isolated at different time points and repeated at least three times.

Determination of degree of conservation of epitopes

ORF 1b nucleotide sequences from a diverse sample of type-II PRRSV isolates (n=34) obtained from NCBI database were translated and aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Regions of ORF1b homologous to peptides containing candidate T-cell epitopes were identified. WebLogo bioinformatics tool was also used to show the graphical representation of the multiple sequence alignment

performed for all the 34 NA sequences (Crooks et al., 2004; Schneider and Stephens, 1990). Moreover, ORF 1b nucleotide sequences from a diverse sample of type-II PRRSV isolates (n=65, Genbank accession numbers pending) were translated, aligned using MUSCLE (Edgar, 2004) and visualized using JALVIEW(Waterhouse et al., 2009). Regions of ORF1b homologous to peptides containing candidate T-cell epitopes were identified and the proportion of PRRSV isolates containing sequences identical to those of the peptide determined as an indicator of conservation within this sample of PRRSV isolates.

Bioinformatics approach

All the three proteins of the PRRSV, namely NSP 9, NSP 10 and NSP 11 were analyzed for the MHC class I and MHC class II-peptide binding prediction tools. BIMAS was used to analyze CTL epitope by binding to the selected overlapping peptides against 33 human alleles present in its database (Parker et al., 1994). The binding affinity ($T_{1/2}$ value) depends on the half-time of dissociation of the β2 micro globulin from HLA. A cutoff $T_{1/2}$ value of ≥ 100 minutes as well as <100 minutes were selected for analysis. SYFPEITHI (Rammensee et al., 1999) and PROPRED (Singh and Raghava, 2001) are the other two algorithms used to identify CTL and class II HLA alleles, respectively. The optimal value for SYFPEITHI (Rammensee et al., 1999) is ≥15 and for PROPRED (Singh and Raghava, 2001) the threshold percent is recommended in between 1-3%. The PROPRED threshold percentage was altered differently to check the binding affinity of the peptides to the MHC class II alleles.

Chapter 4: Results

Screening of PRRSV NSP 9 and NSP 10 for T-cell epitopes using T-cell proliferation assay

Twenty peptide pools from NSP 9 were used for the first round of peptide screening (figure 2). Based on the criteria described in the materials and methods for analysis of functional assays, four out of twenty NSP 9 peptide pools encompassing amino acid positions 103-143, 135-175, 199-239 and 519-559 were identified with stimulation indices at least twice background (Table 2a). As a result of the T-cell proliferation assay of the individual peptides present in those four NSP 9 peptide pools (figure 3a), it was found that four peptides beginning with amino acid positions 119, 151, 207 and 519 elicited positive proliferation results (Table 3).

Fourteen peptide pools from NSP 10 were used for the first round of peptide screening (figure 2b). Two out of fourteen NSP 10 peptide pools, encompassing amino acid positions 129-169 and 193-233, were identified which elicited positive stimulation indices (Table 2). As a result of the T-cell proliferation assay of the individual peptides present in those two NSP 10 peptide pools (figure 3b it was found that two peptides encompassing amino acid positions 209-225 and 217-233 respectively exhibited positive stimulation indices (Table 3).

Seven peptide pools from NSP 11 were used for the first round of peptide screening (figure 2c). Two out of seven peptide pools, encompassing amino acid positions 1-41 and 33-73, respectively exhibited positive stimulation indices (Table 3). The second round of screening was not performed for NSP 11 due to limited number of cryopreserved vials

which could be used for one more set of experiment. We used those cryopreserved PBMCs for IFN-γ secreting ELISpot assay to detect the immunogenic epitopes present in the NSP 11 PRRSV protein. Another reason to directly opt for this immunogenic assay was that we have to detect T-cell epitopes from eight peptides present in those two peptide pools obtained in the first round of screening. Therefore, the one set of cryopreserved PBMCs were directly used for ELISpot assay, particularly in the case of NSP 11.

None of the PBMCs isolated from the control pigs responded to the specific peptides obtained after two rounds of T-cell peptide screening. All the above experiments were performed using fresh PBMCs except NSP 11 which was performed using cryopreserved PBMCs.

Identification of PRRSV immunogenic individual peptides from NSP 9, NSP 10 and NSP 11

Table 4 provides a summary of the results obtained from cryopreserved PBMCs stimulated with NSP 9, 10 and 11 peptides inducing IFN-γ. Out of 4 NSP 9 peptides tested (those that gave a positive response in the lymphoproliferation assay), 2 peptides at amino acid position 119-135 and 151-167 (KEEIALSAQIIQACDIR and VRGNPERVKGVLQNTRF) showed specific immunospots as compared with their non-immunized counterparts (Table 4). During this analysis, the remaining two peptides did not stimulate IFN-γ response in PBMC populations. In case of NSP 10, both the individual peptides stimulated proliferation of IFN-γ secreting cells at different time

points (Table 4). The peptide at amino acid position 209-225 (VRILAGGWCPGKNSFLD) showed significant increase in IFN-γ ELISpot positive cells following stimulation. The peptide at amino acid position 217-233 (CPGKNSFLDEAAYCNHL) was able to show specific immunospots following the second booster dose (121 days post-initial infection and 7 days after second booster). Three out of eight NSP 11 peptides showed significant IFN-γ secretion. These peptides are located at amino acid position 1-17 (GSSSPLPKVAHNLGFYF), 9-25 (VAHNLGFYFSPDLTQFA) and 57-73 (VHKYSRACIGAGYMVGP) (Table 4).

Conservation of PRRSV NSP 9, NSP 10 and NSP 11 peptides identified throughout North American PRRSV Type-II strains

Thirty-four sequences of North American PRRSV isolates obtained from worldwide locations (source: NCBI databank) were aligned using Clustal W analytical tool. Figure 4 illustrates the conservation of these identified immunodominant peptides obtained from NSP 9, NSP 10 and NSP 11 of PRRSV. NSP 9 peptide sequence KEEIALSAQIIQACDIR of FL-12 strain at amino acid position 119 was conserved throughout the North American isolates (figure 4a,b). Two alterations were found at amino acid positions 122, 126 and 133 (valine for isoleucine, valine/Methylamine for alanine and aspartic acid to glycine). In case of peptide sequence VRGNPERVKGVLQNTRF, six alterations were found at amino acid position 151 (valine by isoleucine), 154 (asparagine by aspartic acid), 156 (glutamic acid to aspartic acid), 158 (valine by alanine), 161 (valine by leucine) and 163 (glutamine by lysine)(figure 4a). Conservation variation was also noted in NSP 10 peptide sequence

VRILAGGWCPGKNSFLD at amino acid position 211 where isoleucine was replaced by threonine. In addition, a unique non-conserved region was also noticed in NSP 11 peptide sequence GSSSPLPKVAHNLGFYF where tyrosine was replaced by histidine at amino acid position number 16. Similarly, in case of the other two peptide sequences of NSP 11 amino acid replacement could also be seen (figure 4a). A graphical representation of the T-cell epitopes from PRRSV NSPs 9, 10 and 11 showing 90-95% conservation pattern among 34 NA sequences of PRRSV has been represented in figure 4b.

Table 6 illustrates the conservation of these identified NSP 9, NSP 10 and NSP 11 peptides in a sample of diverse PRRSV field isolates. The NSP 9 peptide sequence of FL-12 strain at amino acid position 119 was conserved in 22 % of the strains throughout the North American isolates (Table 6). Unlike, the second epitope at amino acid position 151 found in NSP 9 sequence was conserved in 84% of the 65 NA-PRRSV sequences analyzed. In case of NSP 10, two T-cell epitopes at amino acid positions 209 and 217 were found to be 95% and 96% conserved, respectively (Table 6). Similarly, NSP 11 T-cell epitopes at amino acid positions 1, 9 and 57 were found to be 96%, 92% and 20% conserved, respectively (Table 6).

Binding specificity of peptides to HLA class I and II molecules predicted by various algorithms

The HLA-peptide binding analysis of PRRSV NSPs 9, 10 and 11 have been carried out at various binding affinities (BIMAS-$T_{1/2}$1-100 minutes; SYFPEITHI-score ≥15 and PROPRED threshold percentage >3%). The peptides showing both proliferation and

immunogenicity properties were selected for such an analysis and have been shown in the Table 5. It was found that one out of two NSP 9 peptides, one out of two NSP 10 peptides and two out of three NSP 11 peptides were able to show good binding affinity in all the three algorithms used. Three peptides showed distinct results by not binding to any of the HLAs present in at least one of the three different algorithms used in this study. NSP 9 peptide at amino acid position 119 could only bind MHC alleles at a low $T_{1/2}$ value (1-50 minutes). It showed, however, good HLA binding affinity by using SYFPEITHI as well as PROPRED at their desired optimum values. Similarly, binding specificity of NSP 10 peptide at amino acid position 217 to MHC class II alleles could be seen only above the threshold value (10%). NSP 11 peptide at amino acid position 1 was unable to bind to both BIMAS and PROPRED at their proposed optimum values. Moreover, it showed binding affinity to the MHC class I and II alleles at a lower $T_{1/2}$ value (1-20 minutes) in BIMAS and 7% threshold value using PROPRED (Table 5). Altogether, these results suggest a good correlation link between the bioinformatics approach and functional assays performed.

Chapter 5: Discussion

Both neutralizing antibodies and CMI contribute towards the establishment of PRRSV protective immunity (Kimman et al., 2009; Lopez and Osorio, 2004). While the mechanisms and viral determinants of protective immunity against PRRSV are incompletely characterized, protection likely results from recognition of viral epitopes by both antibodies and T-cells. Identification of T-cell epitopes mediating heterologous protection, and the nature of variation in those epitopes will directly enhance prediction of vaccine efficacy and guide the rational design or selection of broadly protective vaccines through estimation of the optimal composition of epitopes for the formulation of vaccines efficacious against the majority of PRRSV strains. This would represent a significant advance in the control of PRRSV. Our present report deals with functional T-cell epitope mapping focused on selected nonstructural PRRSV proteins. These NSPs constitute fundamental, highly conserved proteins used for the early stages of viral replication.

The strictly functional approach that we followed in this study has been based on two bonafide CMI assays: lymphocyte proliferation and frequency of IFN-γ secreting cells. It has been reported that lymphocyte proliferation from PRRSV-infected pigs can be detected starting at 4 weeks PI (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). Those proliferating cells can also secrete IFN-γ and hence, T-cell response measured by IFN-γ secreting cell frequency has been shown to be central in clearing viral infections (Callan et al., 1996; Gruters et al., 2002; Martin et al., 2004; Riddell et al., 1991; Riddell et al., 1992).

The emphasis on PRRSV T-cell epitope research and identification has centered, until now, exclusively on important structural components of this virus. Several papers have been published suggesting the existence of T-cell epitopes in GP5 (Diaz et al., 2009; Vashisht et al., 2008), GP4 (Diaz et al., 2009), N (Diaz et al., 2009) and M protein (Wang et al., 2011). In our case we selected three PRRSV NSPs (NSP 9,the RdRp, NSP 10, a helicase and NSP 11, a endonuclease) based on their functional role in viral replication and virulence, (Fang and Snijder, 2010). NSP 9, NSP 10 and NSP 11 seem to be highly conserved, being consistent throughout the North American PRRSV strains, thus constituting good tentative candidates for T-cell epitope mapping to provide cross-protection immunity against PRRSV infection.

Our present report deals with functional epitope mapping focused on selected nonstructural PRRSV proteins. These NSPs constitute fundamental, highly conserved proteins used for the early stages of viral replication. The conservation of the identified epitopes was investigated by aligning amino acid sequences of the NSP 9, NSP 10 and NSP 11 of North American genotype-II PRRSV strains. Considering the above points, our data suggests that T-cell epitope amino acid sequence identity is 91% in NSP 9 (amino acid position 119), 79% in NSP 9 (amino acid position 151), 100% in NSP 10 (amino acid position 209 and 217), 94% in NSP 11 (amino acid position 1), 88% in NSP 11 (amino acid position 9) and 79% in NSP 11 (amino acid position 57) (figure 4). Also, the conservation of the identified epitopes was investigated by aligning sixty-five amino acid sequences of the NSP 9, NSP 10 and NSP 11 of North American genotype-II PRRSV strains. Considering the above points, our data suggests that the T-cell epitopes herein identified range in amino acid sequence identity among 22% in NSP9 (amino acid

position 119), 84% in NSP 9 (amino acid position 151), 95% in NSP 10 (amino acid position 209), 97% in NSP 10 (amino acid position 217), 96% in NSP 11 (amino acid position 1), 92% in NSP 11 (amino acid position 9) and 20% in NSP 11 (amino acid position 57) (Table 6). From these results, we can confirm that even though these epitopes are not absolutely conserved, they remain conserved in most of the North American isolates except for a few mutations in a very low percentage of the strains.

The virus used in our study is the PRRSV FL-12, a highly pathogenic strain derived from an infectious clone constructed upon parental strain NVSL 97-7895, which belongs to North American type-II genotype (Truong et al., 2004). Pigs were infected with the PRRV FL-12 and PBMCs obtained from those pigs were used to analyze synthetic peptides from NSP 9, NSP 10 and NSP 11. From the present data we cannot infer which type of cells, within the reacting PBMCs (i.e. CD4+ vs. CD8+ T-cells) are actually responding to these peptides. Future studies oriented towards such identification would contribute to a more complete understanding on the specific mechanisms on which the development of future vaccines should rely.

An important aspect of this preliminary T-cell epitope identification is the immunogenic character of these epitopes, which has been confirmed by ELISpot. In most cases, the ELISpot results (Table 4) were obtained only after the first or second booster inoculations following the initial infection with PRRSV FL-12. It has been shown that the amount of IFN-γ produced per T-cell against PRRSV is low during the first few weeks after immunization or infection with PRRSV, although it gradually increases significantly after 3-4 months post-vaccination (Meier et al., 2003; Zuckermann et al., 2007). Evidence for a role of CMI in protective immunity against PRRSV has been reported by studies in which

a correlation was found between the IFN-γ response and protective immunity (Meier et al., 2003; Zuckermann et al., 2007). We have previously reported that the IFN-γ response can be used as an indicator of protective immunity and emphasized that in certain cases the post-challenge recall kinetics of serum neutralizing antibodies does not correlate with protection(Zuckermann et al., 2007). Similarly, others have concluded that the presence of virus-specific IFN-γ-secreting T-cell response indicates that vaccination has elicited protective immunity (Diaz et al., 2006; Martelli et al., 2009).

Overall the IFN-γ response observed with these two sets of peptides would represent a recall response mediated by PRRSV-specific memory T-cells (Vashisht et al., 2008). The peptides were synthesized with high purity which explains that the strong reactivity to these peptides was not due to any kind of contamination; rather, it represents PRRSV NSP 9, NSP 10 and NSP 11 immunogenic epitopes. The mean ± standard deviation criterion provides a positive feedback for selecting the peptides with certain cut-off values thereby restricting the selection of the immunodominant T-cell epitopes. Unpublished data from our laboratory have shown that cryopreserved PBMCs and PBMCs extracted from fresh blood exhibit similar activity in ELISpot assays. Although the magnitude of the immune response was different with different peptides, the quantitative results remain truly identical. The different PBMCs used for the comparative ELISpot studies were obtained during the same course of experiments reported here. The PBMCs were cryopreserved at different time points and the results shown in Table 4 are a compiled version of all of them. A thorough and consistent report has been obtained throughout the ELISpot experimental analysis.

The reports of PRRSV T-cell epitopes existing in the literature include different approaches ranging from primarily bioinformatics discovery using algorithms for prediction of T-cell epitopes (Diaz et al., 2009) to those that, like the one herein described, predominantly map T-cell epitopes by scanning of overlapping peptides along the protein sequence and assessing their activity in functional assays (Vashisht et al., 2008; Wang et al., 2011).

It could be argued that false positive and negative results might occur due to the usage of elevated peptide concentrations to stimulate PBMCs in our experimental study (10µg/ml). However, the concentration we used is well within the range of recommended peptide concentration for T-cell epitope mapping and is consistent with those commonly used and reported in recent literature (Assarsson et al., 2008; Dow et al., 2008; Gerner et al., 2009; Haghighi et al., 2009; Streeck et al., 2009; Wulf et al., 2009). False positive peptides are readily excluded by subsequent confirmatory tests. False negatives are reduced by designing pools such that each peptide is contained in more than one pool, minimizing the probability that highly avid peptides will outcompete any individual peptide (Hoffmeister et al., 2003).

ELISpot assay has proved to be a cost-effective technique to detect T-cell epitopes in various viral diseases (Streeck et al., 2009). It should be born in mind, however, that the relationship between the SLA class I and II haplotypes, and the IFN-γ secretion against PRRSV has also not yet been characterized so far. Future studies should focus on the characterization, not only of the type of T-cell prevailing in this epitope stimulation, but also on the characterization of the SLA haplotypes involved in this response. It should be noted that the response that we measured against individual peptides has been observed,

in the best case in ≤ 50 % of the animals (Tables 3 and 4). This is might be expected, given the known polymorphism of the SLA and the fact that pigs used in these experiments, though from a similar genetic background, were of unknown SLA haplotypes.

Taken together, we have identified T-cell epitopes present in the NSP 9, NSP 10 and NSP 11 proteins of PRRSV based on their functional and structural constraints. These proteins were able to show significant T-cell proliferation as well as IFN-γ secretion. The degree of conservation of these epitopes suggests that they may be highly useful in the rational design of broadly efficacious vaccines against PRRSV.

Table1. Name of the peptide pools and individual peptides used for T-cell proliferation and ELISpot assays.

Peptide pool no.	I	II	III	IV	V	VI	VII	VIII	IX	X
NSP9 Peptide name	7-23	39-55	71-87	103-119	135-151	167-183	199-215	231-247	263-279	295-311

	15-31	47-63	79-95	111-127	143-159	175-191	207-223	239-255	271-287	303-319
	23-39	55-71	87-103	119-135	151-167	183-199	215-231	247-263	279-295	311-327
	31-47	63-79	95-111	127-143	159-175	191-207	223-239	255-271	287-303	319-335
	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
	327-343	359-375	391-407	423-439	455-471	487-503	519-535	551-567	583-599	615-631
	335-351	367-383	399-415	431-447	463-479	495-511	527-543	559-575	591-607	623-639
	343-359	375-391	407-423	439-455	471-487	503-519	535-551	567-583	599-615	631-646
	351-367	383-399	415-431	447-463	479-495	511-527	543-559	575-591	607-623	

Peptide pool no.	I	II	III	IV	V	VI	VII	VIII	IX	X
NSP10 Peptide	1-17	33-49	65-81	97-113	129-145	161-177	193-209	225-241	257-273	289-305
	9-25	41-57	73-89	105-121	137-153	169-185	201-217	233-249	265-281	297-313
	17-33	49-65	81-97	113-129	145-161	177-193	209-225	241-257	273-289	305-321
	25-41	57-73	89-105	121-137	153-169	185-201	217-233	249-265	281-297	313-329
	XI	XII	XIII	XIV						
	321-337	353-369	385-401	417-433						
	329-345	361-377	393-409	425-441						
	337-353	369-385	401-417							
	345-361	377-393	409-425							

Peptide pool no.	I	II	III	IV	V	VI	VII
NSP11 Peptide	1-17	33-49	65-81	97-113	129-145	161-177	193-209
	9-25	41-57	73-89	105-123	137-153	169-185	201-217
	17-33	49-65	81-97	113-129	145-161	177-193	209-225
	25-41	57-73	89-105	121-137	153-169	185-201	

Table 2. Identification of peptide pools using lymphoproliferative [H^3] incorporation assay.

protein name	peptide pool no.	overlapping aa sequence in a peptide pool	no. of resp. pigs for each peptide pool
NSP 9	IV	NTGIDGTLWDFEAEAT**KEEIALSAQIIQACDIR**RGDAPEIG	3\8
	V	RRGDAPEIGLPYKLYP**VRGNPERVKGVLQNTRF**GDIPYKTP	1\8
	VII	RSVLATTM**PSGFELYVPTIPASVLDYLD**SRPDCPKQLTEHG	1\8
	XVII	**ESPTMPNYHWWVEHLNL**MLGFQTDPKKTAITDSPSFLGCRI	1\8
NSP 10	V	CKEINMVAVASNVLRSRFIIGPPGAGKTYWLLQQVQDGDVI	4\4
	VII	AGTTLQFPAPSRTGPW**VRILAGGWCPGKNSFLDEAAYCNHL**	3\4
NSP 11	I	**GSSSPLPKVAHNLGFYFSPDLTQFA**KLPVELAPHWPVVTTQ	1\4
	II	PHWPVVTTQNNEKWPDRLVASLRP**VHKYSRACIGAGYMVGP**	1\4

Notes: Pigs in the principal group were inoculated with PRRSV FL-12 and blood samples were collected starting at 7 d.p.i. The results shown here is the average of the PBMCs collected on 14 d.p.i., 21 d.p.i. and 28 d.p.i. This table shows the nine amino acid overlapping sequence length of peptide pools identified from PRRSV NSP 9, 10 and 11 at their respective amino acid positions after the first round of T-cell epitope mapping. The number of responding pigs is the immunized animals whose PBMC exhibited a peptide-specific proliferation with a stimulation index ≥ 2 along with a zero background response. The response to the peptides had to be ≥ 2 than the proliferated cells of non-immunized control pigs to be considered positive. None of the PBMCs samples isolated from the control pigs responded to the peptides pools herein shown.

Table 3. Identification of heptadecamer PRRSV peptides likely to contain of NSP 9 and NSP 10 epitopes.

protein name	peptide name	peptide sequence	no. of resp. pigs for each individual peptide
NSP 9	119-135	KEEIALSAQIIQACDIR	4\8
	151-167	VRGNPERVKGVLQNTRF	2\8
	207-223	PSGFELYVPTIPASVLD	1\8
	519-535	ESPTMPNYHWWVEHLNL	2\8
NSP 10	209-225	VRILAGGWCPGKNSFLD	2\4
	217-233	CPGKNSFLDEAAYCNHL	2\4

Notes: Pigs in the principal group were inoculated with PRRSV FL-12 and blood samples were collected 7 days post first booster dose (boosters were applied at 21 d.p.i for NSP 9 study and at 57 d.p.i for NSP 10 study). This table shows the heptadecamer sequence length of the individual peptides identified from PRRSV NSP 9 and 10 at their respective amino acid positions after the second round of T-cell epitope mapping. The number of responding pigs is the immunized animals whose PBMC exhibited a peptide-specific proliferation with a stimulation index ≥ 2 along with a zero background response. The response to the peptides had to be ≥ 2 than the proliferated cells of non-immunized pigs to be considered positive. None of the PBMCs samples isolated from the non-immunized control pigs responded to any of these individual peptides.

Table 4. T-cell epitopes in PRRSV NSP 9, NSP 10 and NSP 11 identified by the IFN-γ induction criteria.

Protein name	aa position	peptide sequence	[a]max. resp	[b]tot. resp	[c]avg. resp	[d]no. resp. pigs	[e]avg. resp pigs
NSP 9	**119-135**	**KEEIALSAQIIQACDIR**	**8**	**10.6**	**1.7**	**2\8**	**12.8**
NSP 9	**151-167**	**VRGNPERVKGVLQNTRF**	**8. 6**	**5.3**	**0.8**	**2\8**	**8.5**
NSP 9	207-223	PSGFELYVPTIPASVLD	3.7	9	1.5	1\8	0.7
NSP 9	519-535	ESPTMPNYHWWVEHLNL	2.7	3	0.5	2\8	4.7
NSP 10	**209-225**	**VRILAGGWCPGKNSFLD**	**7.3**	**4.3**	**1.0**	**1\4**	**14**
NSP 10	**217-233**	**CPGKNSFLDEAAYCNHL**	**27.3**	**33.9**	**8.4**	**2\4**	**21.2**
NSP 11	**1-17**	**GSSSPLPKVAHNLGFYF**	**37.33**	**63.99**	**21.33**	**1\4**	**65**
NSP 11	**9-25**	**VAHNLGFYFSPDLTQFA**	**40**	**48.99**	**16.33**	**1\4**	**67.67**
NSP 11	**57-73**	**VHKYSRACIGAGYMVGP**	**39.66**	**63.33**	**21.11**	**1\4**	**65.33**

Notes: Pigs were initially infected with PRRSV FL-12 and subsequently were administered two additional boosters (at 21 d.p.i and 96 d.p.i for the NSP 9 experiment, and at 57 and 114 d.p.i for the NSP 10 and NSP 11 experiments. The ELISpot results shown here is the compilation of data obtained from the interferon response from the PBMCs cryopreserved at different time points post first and second booster doses. The ELISpot experiments for each protein were performed independently.

[a]Max.resp: number of IFN-producing cells detected in PBMC from the highest responder pig among all 2 PBMC samples tested.
[b]Tot.resp: sum of all of the IFN-producing cells (minus background) detected in the 2 individually tested PBMC samples.
[c]Avg. resp: sum of all the IFN-producing cells (minus background) detected in the 2 PBMC samples tested divided by 2, the number of pigs tested.
[d]No. Resp. pigs: number of pigs exhibiting a frequency of peptide-specific IFN-γ secreting cells with a stimulation index ≥ 2 than the cells only. If background response was zero the response to the peptide had to be ≥2 to be considered positive.
[e]Avg Resp pigs: average of the peptide-specific IFN-specific IFN-response (minus background) of all the pigs exhibiting a response to the individual peptide.
[f]Mean±Std.D: mean ± standard deviation of the values shown for each of the four T-cell peptide candidate for each of the selection criteria were concluded to contain an immunodominant peptide (Vashisht et al., 2008).

Peptide sequences in bold represent the positive peptides which showed maximum IFN-γ secretion. Controls used in this assay were: concanavalin A (positive control), FL-12 infected cell homogenates (positive control) and PBMCs only, without peptide (negative control). None of these peptides reacted with the PBMCs of the non-immunized control pigs (mean ± std.dev. = 0±3).

Table 5. Feasibility of PRRSV nonstructural proteins to HLA using various peptide prediction tools at different threshold values.

Protein name	aa posn.	BIMAS		SYFPEITHI	PROPRED	
		At low $T_{1/2}$(<100)	At $T_{1/2}$(≥100)	score ≥15	Threshold (1-3%)	Above threshold
NSP9	119	+++	no	+++	+	-
	151	+++	++	+++	+++	-
NSP10	209	+++	+	+++	+++	-
	217	+++	+	++	no	+
NSP11	1	+++	no	+++	no	+
	9	+++	++	+++	+++	-
	57	+++	++	+++	+++	-

Note: Plus sign represents the different HLA alleles bound to T-cell epitopes obtained from PRRSV NSPs 9, 10 and 11. '+'=one allele; '++'=2-3 alleles; '+++'=3 or more alleles; 'no'=no alleles binding.

Table 6. Conserved T-cell epitopes in NSP 9, NSP 10 and NSP 11.

Sequence	Count	*Proportion
NSP 9 (119-135)		
KEEVALSAQIIQACDIR	28	0.43
KEEIALSAQIIQACDIR	14	0.22
KEEIALSAQIIQACGIR	8	0.12
KEEIALSAQIIQACDMR	5	0.08
KEEVALSAQIIQACDMR	2	0.03
KEEVALSAQIIQACGIR	2	0.03
KEEIELSAQIIQACGIR	2	0.03
KEEIALSTQIIQACDIR	1	0.02
KEEIALSAQIIQACSIR	1	0.02
KEEIALSEQIIQACDIR	1	0.02
KEEVALSTQIIQACDIR	1	0.02
NSP 9 (151-167)		
VRGNPERVKGVLQNTRF	55	0.8461538
IRGNPERVKGVLRNTRF	1	0.0153846
VRDNPERVKGVLKNTRF	1	0.0153846
VRGDPERVKGVLKNTRF	1	0.0153846
VRGNPERARGVLMNTRF	1	0.0153846
VRGNPERVNGVLQNTRF	2	0.0307692
VRGNPERVKGVLRNTRF	3	0.0461538
VRGNPERVKGVLKNTRF	1	0.0153846
NSP 10 (209-225)		
VRILAGGWCPGKNSFLD	62	0.9538462
VRILAGGWCPGRNSFLD	2	0.0307692
VRILAGRWCPGKNSFLD	1	0.0153846
NSP 10 (217-233)		
CPGKNSFLDEAAYCNHL	63	0.9692308
CPGRNSFLDEAAYCNHL	2	0.0307692
NSP 11 (1-17)		
GSSSPLPKVAHNLGFYF	63	0.96923077
NSP 11 (9-25)		
VAHNLGFYFSPDLTQFA	60	0.92307692
VAHNLGFYFSPDLIQFA	2	0.03076923
VAHNLGFYFSPDLAQFA	1	0.01538462
NSP 11 (57-73)		
VHKYSRACIGAGYMVGP	13	0.2

IHKYSRACIGAGYMVGP	44	0.67692308
IHKHSRACVGAGYMVGP	2	0.03076923
IHKHSRACIGAGYMVGP	2	0.03076923
IHKYSRACVGAGYMVGP	1	0.01538462
IHNYSRACIGAGYMVGP	1	0.01538462

Notes: Sequences in bold define the T-cell epitopes obtained in our study after stringent categorization of the heptadecamer peptides present in NSP 9, NSP 10 and NSP 11. Other heptadecamer sequences were considered from various other PRRSV isolates among the 65 sequences aligned. The count number denotes the number of identical sequences out of 65 sequences aligned using JALVIEW. *Proportion of the sequence represents the conservation of those T-cell epitopes by applying the following formula:

no. of count of similar sequences/total no. of sequences used for multiple alignment.

Figure 1

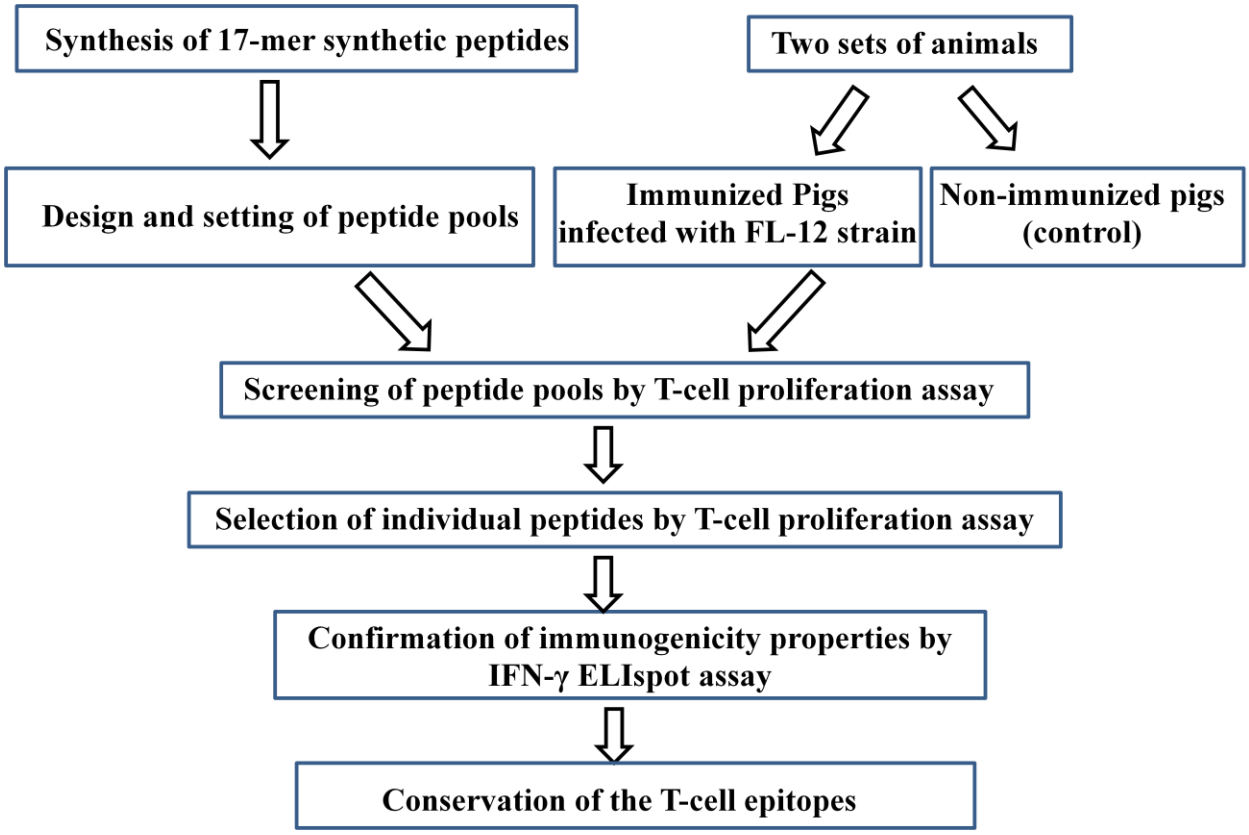
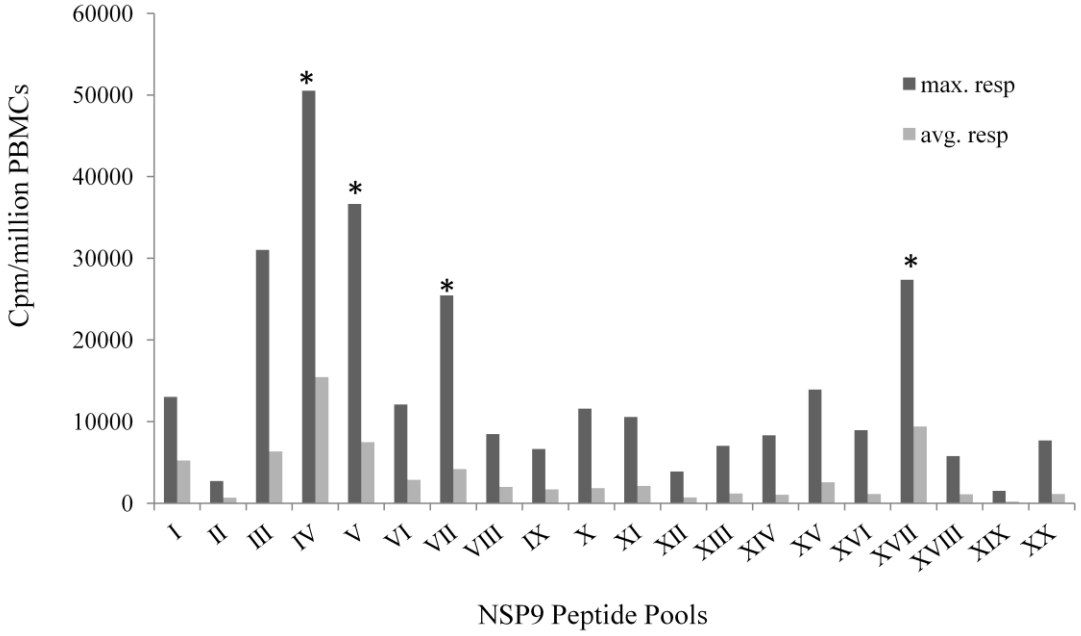

Synthesis of 17-mer synthetic peptides
Two sets of animals
Design and setting of peptide pools
Immunized Pigs infected with FL-12 strain
Non-immunized pigs (control)
Screening of peptide pools by T-cell proliferation assay
Selection of individual peptides by T-cell proliferation assay
Confirmation of immunogenicity properties by IFN-γ ELIspot assay
Conservation of the T-cell epitopes

Figure 2

(a)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30000
20000
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Cpm/million PBMCs
max. resp
avg. resp
*
*
*
*
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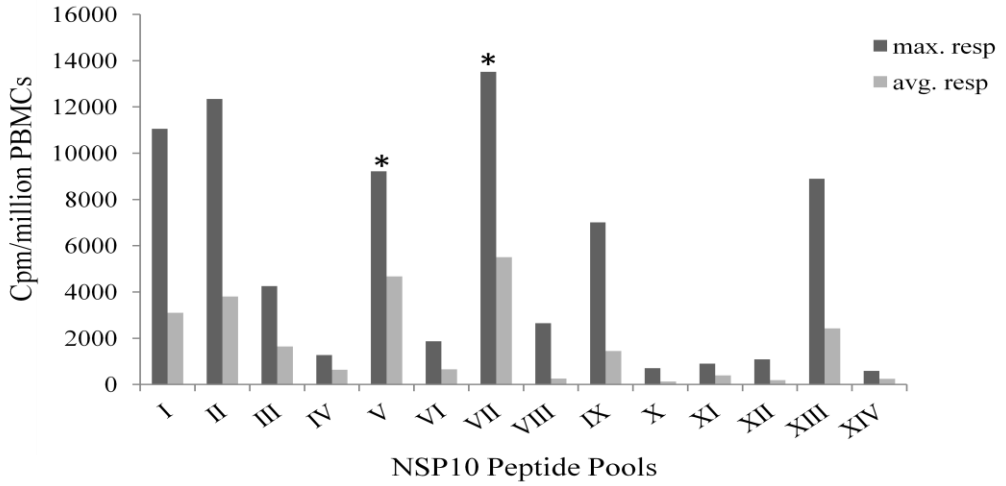
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
NSP9 Peptide Pools

(b)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Cpm/million PBMCs
max. resp
avg. resp
*
*
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III
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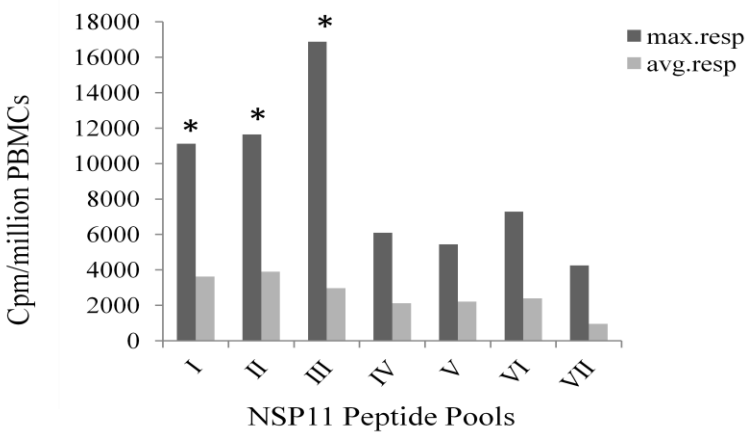
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
NSP10 Peptide Pools

(c)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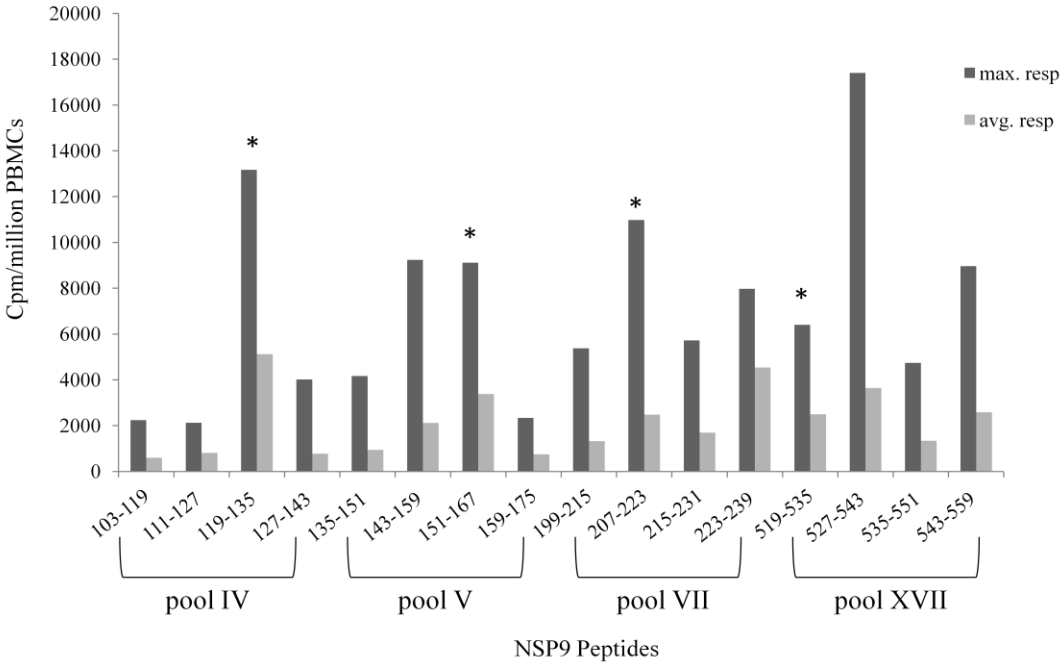
0
Cpm/million PBMCs
*
*
*
I
II
III
IV
V
VI
VII
NSP11 Peptide Pools
max.resp
avg.resp

Figure 3

(a)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199-215
207-223
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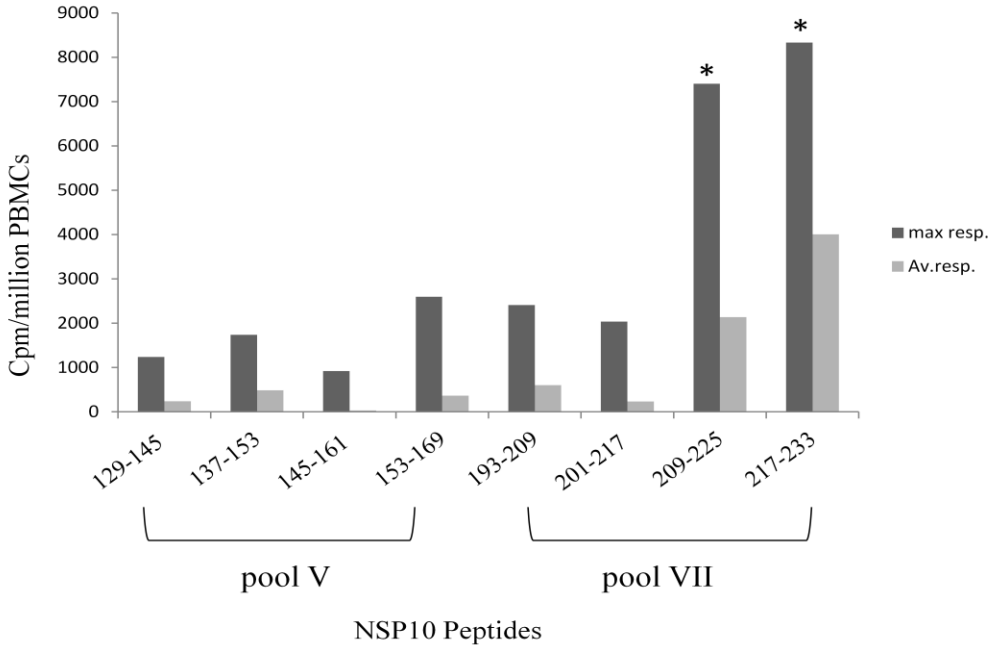
pool IV
pool V
pool VII
pool XVII
NSP9 Peptides

(b)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max resp.
Av.resp.
*
*
129-145
137-153
145-161
153-169
193-209
201-217
209-225
217-233
pool V
pool VII
NSP10 Peptides

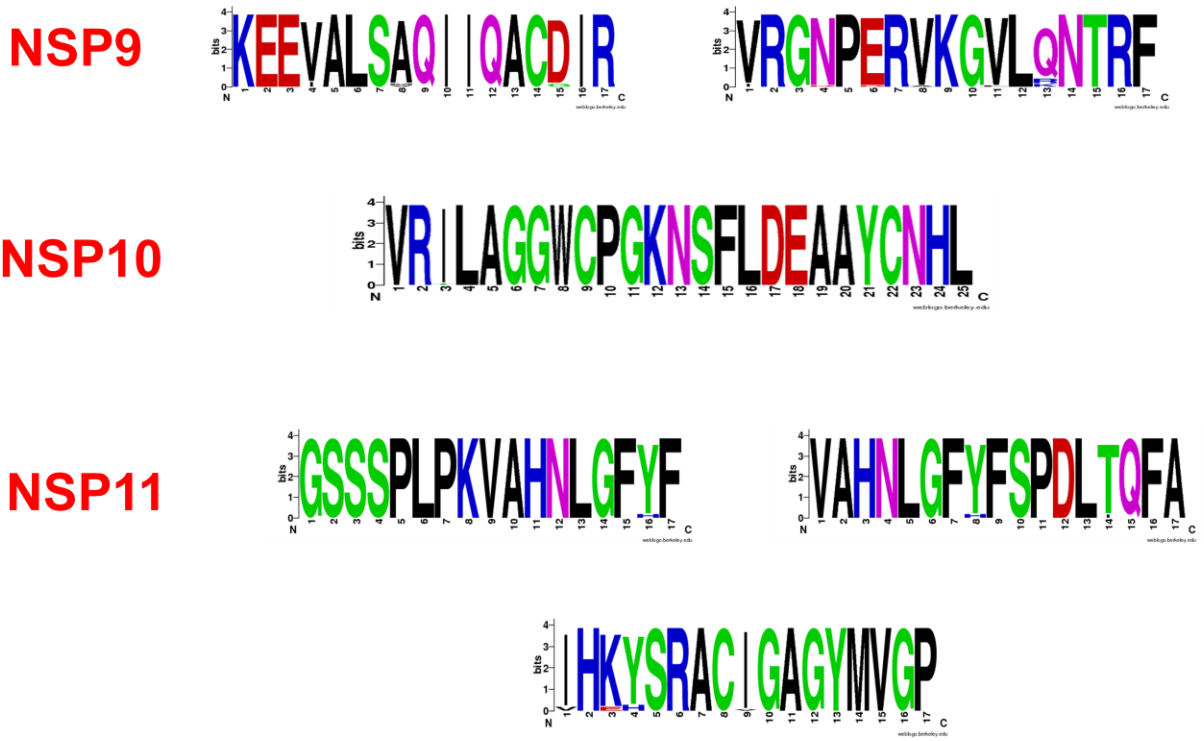
Figure 4

(a)

PRRSV proteins	NSP 9 SEQUENCE		NSP 10 SEQUENCE		NSP 11 SEQUENCE		
aa length / Acsn. no.	119-135	151-167	209-225	217-233	1-17	9-25	57-73
ACT78500.1	KEEVALSAQIIQACDIR	IRGNPERVKGVLRNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHEYSRACIGAGYMVGP
AAF65934.1	KEEVALSAQIIQACDIR	IRGNPERVKGVLRNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHEYSRACIGAGYMVGP
AAD27652.2	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
AAY53872.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACY29599.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACP43263.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
AAU20775.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFHF	VAHNLGFHFSPDLTQFA	IHKYSRACIGAGYMVGP
AAT00223.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFHF	VAHNLGFHFSPDLTQFA	IHKYSRACIGAGYMVGP
ACU01779.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
AAD12125.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACU01790.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ABU43301.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
BAG49669.1	KEEVALSMQIIQACDIR	VRGDPERVKGVLRNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKHSRACVGAGYMVGP
ACN93869.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACN93853.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACN93861.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACT82258.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACT82250.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACQ71961.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACV95340.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACV91358.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACV91374.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACW82438.1	KEEIALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACP43579.1	KEEIALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACQ71968.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRTLAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACZ58646.1	KEEVALSVQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACZ54919.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACU31101.1	KEEVALSAQIIQACDIR	VRGNPDRVKGLLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACT33426.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ACF94577.1	KEEVALSAQIIQACDIR	VRGNPERAKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	IHKYSRACIGAGYMVGP
ABU43166.1	KEEIALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	VHKYSRACIGAGYMVGP
AAS59261.1	KEEIALSAQIIQACDIR	VRGNPERVKGVLQNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLTQFA	VHKYSRACIGAGYMVGP
ABU43327.1	KEEVALSAQIIQACGIR	VRGNPERVKGVLKNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLIQFA	IHKHSRACIGAGYMVGP
ABU43310.1	KEEVALSAQIIQACDIR	VRGNPERVKGVLKNTRF	VRILAGGWCPGKNSFLDEAAYCNHL		GSSSPLPKVAHNLGFYF	VAHNLGFYFSPDLIQFA	IHKHSRACIGAGYMVGP
consensus	***:*** ******.**	:**:*:*.**:*:****	** **********************		***************:*	*******:***** ***	:*::****:********

Notes: Sequence accession number in bold red defines the PRRSV virulent strain (FL-12) used in our study to determine T-cell epitopes after stringent categorization of the heptadecamer peptides present in NSP 9, NSP 10 and NSP 11. Other heptadecamer sequences were considered from various other NA-PRRSV isolates among the 34 sequences aligned. The bold letters in red denotes the amino acid variations observed in the different PRRSV sequences used for the multiple sequence alignment.

(b)



Note: Graphical representation showing conservation of the seven T-cell epitopes obtained from NSP 9, 10 and 11 by performing multiple sequence alignment via weblogo bioinformatics tool. The amino acids which reach 4 bits on the y-scale are highly conserved whereas the one which are lower than that are conserved with a lower percentage.

References:-

Albina, E., Carrat, C. and Charley, B. (1998) Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. J Interferon Cytokine Res 18(7), 485-90.

Allende, R., Lewis, T.L., Lu, Z., Rock, D.L., Kutish, G.F., Ali, A., Doster, A.R. and Osorio, F.A. (1999) North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J Gen Virol 80 (Pt 2), 307-15.

Assarsson, E., Bui, H.H., Sidney, J., Zhang, Q., Glenn, J., Oseroff, C., Mbawuike, I.N., Alexander, J., Newman, M.J., Grey, H. and Sette, A. (2008) Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. Journal of virology 82(24), 12241-51.

Bastos, R.G., Dellagostin, O.A., Barletta, R.G., Doster, A.R., Nelson, E., Zuckermann, F. and Osorio, F.A. (2004) Immune response of pigs inoculated with Mycobacterium bovis BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. Vaccine 22(3-4), 467-74.

Bautista, E.M., Faaberg, K.S., Mickelson, D. and McGruder, E.D. (2002) Functional properties of the predicted helicase of porcine reproductive and respiratory syndrome virus. Virology 298(2), 258-270.

Bautista, E.M. and Molitor, T.W. (1997) Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral Immunol 10(2), 83-94.

Bautista, E.M. and Molitor, T.W. (1999) IFN gamma inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Arch Virol 144(6), 1191-200.

Bautista, E.M., Suarez, P. and Molitor, T.W. (1999) T cell responses to the structural polypeptides of porcine reproductive and respiratory syndrome virus. Arch Virol 144(1), 117-34.

Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D. and Chladek, D. (1992) Characterization of Swine Infertility and Respiratory Syndrome (Sirs) Virus (Isolate Atcc Vr-2332). Journal of Veterinary Diagnostic Investigation 4(2), 127-133.

Beura, L.K., Sarkar, S.N., Kwon, B., Subramaniam, S., Jones, C., Pattnaik, A.K. and Osorio, F.A. Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. J Virol 84(3), 1574-84.

Bilodeau, R., Archambault, D., Vezina, S.A., Sauvageau, R., Fournier, M. and Dea, S. (1994) Persistence of porcine reproductive and respiratory syndrome virus infection in a swine operation. Can J Vet Res 58(4), 291-8.

Buddaert, W., Van Reeth, K. and Pensaert, M. (1998) In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV). Adv Exp Med Biol 440, 461-7.

Callan, M.F., Steven, N., Krausa, P., Wilson, J.D., Moss, P.A., Gillespie, G.M., Bell, J.I., Rickinson, A.B. and McMichael, A.J. (1996) Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. Nat Med 2(8), 906-11.

Cano, J.P., Dee, S.A., Murtaugh, M.P. and Pijoan, C. (2007a) Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. Vaccine 25(22), 4382-91.

Cano, J.P., Dee, S.A., Murtaugh, M.P., Trincado, C.A. and Pijoan, C.B. (2007b) Effect of vaccination with a modified-live porcine reproductive and respiratory syndrome virus vaccine on dynamics of homologous viral infection in pigs. Am J Vet Res 68(5), 565-71.

Cavanagh, D. (1997) Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 142(3), 629-33.

Charerntantanakul, W., Platt, R., Johnson, W., Roof, M., Vaughn, E. and Roth, J.A. (2006) Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 109(1-2), 99-115.

Cho, J.G. and Dee, S.A. (2006) Porcine reproductive and respiratory syndrome virus. Theriogenology 66(3), 655-662.

Christianson, W.T., Collins, J.E., Benfield, D.A., Harris, L., Gorcyca, D.E., Chladek, D.W., Morrison, R.B. and Joo, H.S. (1992) Experimental Reproduction of Swine Infertility and Respiratory Syndrome in Pregnant Sows. American Journal of Veterinary Research 53(4), 485-488.

Christopher-Hennings, J., Nelson, E.A., Nelson, J.K. and Benfield, D.A. (1997) Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. Am J Vet Res 58(1), 40-5.

Christopher-Hennings, J., Nelson, E.A., Nelson, J.K., Hines, R.J., Swenson, S.L., Hill, H.T., Zimmerman, J.J., Katz, J.B., Yaeger, M.J., Chase, C.C. and et al. (1995) Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. J Clin Microbiol 33(7), 1730-4.

Christopherhennings, J., Nelson, E.A., Hines, R.J., Nelson, J.K., Swenson, S.L., Zimmerman, J.J., Chase, C.C.L., Yaeger, M.J. and Benfield, D.A. (1995) Persistence of Porcine Reproductive and Respiratory Syndrome Virus in Serum and Semen of Adult Boars. Journal of Veterinary Diagnostic Investigation 7(4), 456-464.

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S. and et al. (1992) Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 4(2), 117-26.

Conzelmann, K.K., Visser, N., Vanwoensel, P. and Thiel, H.J. (1993) Molecular Characterization of Porcine Reproductive and Respiratory Syndrome Virus, a Member of the Arterivirus Group. Virology 193(1), 329-339.

Cooper, V.L., Doster, A.R., Hesse, R.A. and Harris, N.B. (1995) Porcine Reproductive and Respiratory Syndrome - Neb-1 Prrsv Infection Did Not Potentiate Bacterial Pathogens. Journal of Veterinary Diagnostic Investigation 7(3), 313-320.

Crooks, G.E., Hon, G., Chandonia, J.M. and Brenner, S.E. (2004) WebLogo: a sequence logo generator. Genome Res 14(6), 1188-90.

Dea, S., Gagnon, C.A., Mardassi, H. and Milane, G. (1996) Antigenic variability among north American and European strains of porcine reproductive and respiratory syndrome virus as defined by monoclonal antibodies to the matrix protein. Journal of Clinical Microbiology 34(6), 1488-1493.

Dea, S., Gagnon, C.A., Mardassi, H., Pirzadeh, B. and Rogan, D. (2000) Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Archives of Virology 145(4), 659-688.

Delputte, P.L., Costers, S. and Nauwynck, H.J. (2005) Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. J Gen Virol 86(Pt 5), 1441-5.

Delputte, P.L., Meerts, P., Costers, S. and Nauwynck, H.J. (2004) Effect of virus-specific antibodies on attachment, internalization and infection of porcine reproductive and respiratory syndrome virus in primary macrophages. Vet Immunol Immunopathol 102(3), 179-88.

Delputte, P.L., Vanderheijden, N., Nauwynck, H.J. and Pensaert, M.B. (2002) Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. J Virol 76(9), 4312-20.

Depla, E., Van der Aa, A., Livingston, B.D., Crimi, C., Allosery, K., De Brabandere, V., Krakover, J., Murthy, S., Huang, M., Power, S., Babe, L., Dahlberg, C., McKinney, D., Sette, A., Southwood, S., Philip, R., Newman, M.J. and Meheus, L. (2008) Rational design of a multiepitope vaccine encoding T-lymphocyte epitopes for treatment of chronic hepatitis B virus infections. J Virol 82(1), 435-50.

Dewey, C., Charbonneau, G., Carman, S., Hamel, A., Nayar, G., Friendship, R., Eernisse, K. and Swenson, S. (2000) Lelystad-like strain of porcine reproductive and respiratory syndrome virus (PRRSV) identified in Canadian swine. Can Vet J 41(6), 493-4.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. and Mateu, E. (2005) Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. J Gen Virol 86(Pt 7), 1943-51.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J. and Mateu, E. (2006) Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. Virology 351(2), 249-59.

Diaz, I., Pujols, J., Ganges, L., Gimeno, M., Darwich, L., Domingo, M. and Mateu, E. (2009) In silico prediction and ex vivo evaluation of potential T-cell epitopes in glycoproteins 4 and 5 and nucleocapsid protein of genotype-I (European) of porcine reproductive and respiratory syndrome virus. Vaccine 27(41), 5603-11.

Domingo, E., Baranowski, E., Ruiz-Jarabo, C.M., Martin-Hernandez, A.M., Saiz, J.C. and Escarmis, C. (1998) Quasispecies structure and persistence of RNA viruses. Emerg Infect Dis 4(4), 521-7.

Domingo, E. and Holland, J.J. (1992) Complications of RNA heterogeneity for the engineering of virus vaccines and antiviral agents. Genet Eng (N Y) 14, 13-31.

Dow, C., Oseroff, C., Peters, B., Nance-Sotelo, C., Sidney, J., Buchmeier, M., Sette, A. and Mothe, B.R. (2008) Lymphocytic choriomeningitis virus infection yields overlapping CD4+ and CD8+ T-cell responses. Journal of virology 82(23), 11734-41.

Drew, T.W., Meulenberg, J.J., Sands, J.J. and Paton, D.J. (1995) Production, characterization and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome virus. J Gen Virol 76 (Pt 6), 1361-9.

Duan, X., Nauwynck, H.J., Favoreel, H.W. and Pensaert, M.B. (1998) Identification of a putative receptor for porcine reproductive and respiratory syndrome virus on porcine alveolar macrophages. J Virol 72(5), 4520-3.

Duan, X., Nauwynck, H.J. and Pensaert, M.B. (1997) Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). Archives of Virology 142(12), 2483-2497.

Duarte, E.A., Novella, I.S., Weaver, S.C., Domingo, E., Wain-Hobson, S., Clarke, D.K., Moya, A., Elena, S.F., de la Torre, J.C. and Holland, J.J. (1994) RNA virus quasispecies: significance for viral disease and epidemiology. Infect Agents Dis 3(4), 201-14.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5), 1792-7.

Fang, L., Jiang, Y., Xiao, S., Niu, C., Zhang, H. and Chen, H. (2006) Enhanced immunogenicity of the modified GP5 of porcine reproductive and respiratory syndrome virus. Virus Genes 32(1), 5-11.

Fang, Y., Kim, D.Y., Ropp, S., Steen, P., Christopher-Hennings, J., Nelson, E.A. and Rowland, R.R. (2004) Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. Virus Res 100(2), 229-35.

Fang, Y., Schneider, P., Zhang, W.P., Faaberg, K.S., Nelson, E.A. and Rowland, R.R. (2007) Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. Arch Virol 152(5), 1009-17.

Fang, Y. and Snijder, E.J. (2010) The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus Res 154(1-2), 61-76.

Gaudreault, N., Rowland, R.R. and Wyatt, C.R. (2009) Factors affecting the permissiveness of porcine alveolar macrophages for porcine reproductive and respiratory syndrome virus. Arch Virol 154(1), 133-6.

Gerner, W., Hammer, S.E., Wiesmuller, K.H. and Saalmuller, A. (2009) Identification of major histocompatibility complex restriction and anchor residues of foot-and-mouth disease virus-derived bovine T-cell epitopes. Journal of virology 83(9), 4039-50.

Goldberg, T.L., Lowe, J.F., Milburn, S.M. and Firkins, L.D. (2003) Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection. Virology 317(2), 197-207.

Gruters, R.A., van Baalen, C.A. and Osterhaus, A.D. (2002) The advantage of early recognition of HIV-infected cells by cytotoxic T-lymphocytes. Vaccine 20(15), 2011-5.

Haghighi, H.R., Read, L.R., Haeryfar, S.M., Behboudi, S. and Sharif, S. (2009) Identification of a dual-specific T cell epitope of the hemagglutinin antigen of an h5 avian influenza virus in chickens. PLoS ONE 4(11), e7772.

Hoffmeister, B., Kiecker, F., Tesfa, L., Volk, H.D., Picker, L.J. and Kern, F. (2003) Mapping T cell epitopes by flow cytometry. Methods 29(3), 270-81.

Hopper, S.A., White, M.E.C. and Twiddy, N. (1992) An Outbreak of Blue-Eared Pig-Disease (Porcine Reproductive and Respiratory Syndrome) in 4 Pig Herds in Great-Britain. Veterinary Record 131(7), 140-144.

Jiang, W., Jiang, P., Li, Y., Wang, X. and Du, Y. (2007a) Analysis of immunogenicity of minor envelope protein GP3 of porcine reproductive and respiratory syndrome virus in mice. Virus Genes 35(3), 695-704.

Jiang, W., Jiang, P., Wang, X., Li, Y. and Du, Y. (2007b) Influence of porcine reproductive and respiratory syndrome virus GP5 glycoprotein N-linked glycans on immune responses in mice. Virus Genes 35(3), 663-71.

Jiang, W., Jiang, P., Wang, X., Li, Y. and Du, Y. (2008) Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus Res 136(1-2), 50-7.

Jiang, Y., Xiao, S., Fang, L., Yu, X., Song, Y., Niu, C. and Chen, H. (2006) DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. Vaccine 24(15), 2869-79.

Kapur, V., Elam, M.R., Pawlovich, T.M. and Murtaugh, M.P. (1996) Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J Gen Virol 77 (Pt 6), 1271-6.

Katz, J.B., Shafer, A.L., Eernisse, K.A., Landgraf, J.G. and Nelson, E.A. (1995) Antigenic differences between European and American isolates of porcine reproductive and respiratory syndrome virus (PRRSV) are encoded by the carboxyterminal portion of viral open reading frame 3. Vet Microbiol 44(1), 65-76.

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S. and Frey, M.L. (1993) Enhanced Replication of Porcine Reproductive and Respiratory Syndrome (Prrs) Virus in a Homogeneous Subpopulation of Ma-104 Cell-Line. Archives of Virology 133(3-4), 477-483.

Kim, W.I. and Yoon, K.J. (2008) Molecular assessment of the role of envelope-associated structural proteins in cross neutralization among different PRRS viruses. Virus Genes 37(3), 380-91.

Kimman, T.G., Cornelissen, L.A., Moormann, R.J., Rebel, J.M. and Stockhofe-Zurwieden, N. (2009) Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27(28), 3704-18.

Kreher, C.R., Dittrich, M.T., Guerkov, R., Boehm, B.O. and Tary-Lehmann, M. (2003) CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. J Immunol Methods 278(1-2), 79-93.

Kreutz, L.C. and Ackermann, M.R. (1996) Porcine reproductive and respiratory syndrome virus enters cells through a low pH-dependent endocytic pathway. Virus Research 42(1-2), 137-147.

Labarque, G., Reeth, K.V., Nauwynck, H., Drexler, C., Van Gucht, S. and Pensaert, M. (2004) Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. Vaccine 22(31-32), 4183-90.

Lager, K.M., Mengeling, W.L. and Brockmeier, S.L. (1999) Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. Am J Vet Res 60(8), 1022-7.

Lamontagne, L., Page, C., Larochelle, R. and Magar, R. (2003) Porcine reproductive and respiratory syndrome virus persistence in blood, spleen, lymph nodes, and tonsils of experimentally infected pigs depends on the level of CD8(high) T cells. Viral Immunology 16(3), 395-406.

Lawson, S.R., Rossow, K.D., Collins, J.E., Benfield, D.A. and Rowland, R.R.R. (1997) Porcine reproductive and respiratory syndrome virus infection of gnotobiotic pigs: sites of virus replication and co-localization with MAC-387 staining at 21 days post-infection. Virus Research 51(2), 105-113.

Lee, S.M., Schommer, S.K. and Kleiboeker, S.B. (2004) Porcine reproductive and respiratory syndrome virus field isolates differ in in vitro interferon phenotypes. Vet Immunol Immunopathol 102(3), 217-31.

Loemba, H.D., Mounir, S., Mardassi, H., Archambault, D. and Dea, S. (1996) Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch Virol 141(3-4), 751-61.

Lopez Fuertes, L., Domenech, N., Alvarez, B., Ezquerra, A., Dominguez, J., Castro, J.M. and Alonso, F. (1999) Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Res 64(1), 33-42.

Lopez, O.J., Oliveira, M.F., Garcia, E.A., Kwon, B.J., Doster, A. and Osorio, F.A. (2007) Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. Clin Vaccine Immunol 14(3), 269-75.

Lopez, O.J. and Osorio, F.A. (2004) Role of neutralizing antibodies in PRRSV protective immunity. Vet Immunol Immunopathol 102(3), 155-63.

Loving, C.L., Brockmeier, S.L. and Sacco, R.E. (2007) Differential type I interferon activation and susceptibility of dendritic cell populations to porcine arterivirus. Immunology 120(2), 217-29.

Loving, C.L., Brockmeier, S.L., Vincent, A.L., Lager, K.M. and Sacco, R.E. (2008) Differences in clinical disease and immune response of pigs challenged with a high-dose versus low-dose inoculum of porcine reproductive and respiratory syndrome virus. Viral Immunol 21(3), 315-25.

Luo, R., Xiao, S., Jiang, Y., Jin, H., Wang, D., Liu, M., Chen, H. and Fang, L. (2008) Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses interferon-beta production by interfering with the RIG-I signaling pathway. Mol Immunol 45(10), 2839-46.

Mardassi, H., Massie, B. and Dea, S. (1996) Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology 221(1), 98-112.

Mardassi, H., Mounir, S. and Dea, S. (1994) Identification of major differences in the nucleocapsid protein genes of a Quebec strain and European strains of porcine reproductive and respiratory syndrome virus. J Gen Virol 75 (Pt 3), 681-5.

Mardassi, H., Mounir, S. and Dea, S. (1995) Molecular analysis of the ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus, Quebec reference strain. Arch Virol 140(8), 1405-18.

Martelli, P., Cordioli, P., Alborali, L.G., Gozio, S., De Angelis, E., Ferrari, L., Lombardi, G. and Borghetti, P. (2007) Protection and immune response in pigs intradermally vaccinated against porcine reproductive and respiratory syndrome (PRRS) and subsequently exposed to a heterologous European (Italian cluster) field strain. Vaccine 25(17), 3400-8.

Martelli, P., Gozio, S., Ferrari, L., Rosina, S., De Angelis, E., Quintavalla, C., Bottarelli, E. and Borghetti, P. (2009) Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: Clinical protection and cell-mediated immunity. Vaccine 27(28), 3788-99.

Martin, P., Parroche, P., Chatel, L., Barretto, C., Beck, A., Trepo, C., Bain, C., Lone, Y.C., Inchauspe, G. and Fournillier, A. (2004) Genetic immunization and comprehensive screening approaches in HLA-A2 transgenic mice lead to the identification of three novel epitopes in hepatitis C virus NS3 antigen. J Med Virol 74(3), 397-405.

Mateu, E. and Diaz, I. (2008) The challenge of PRRS immunology. Vet J 177(3), 345-51.

Mateu, E., Diaz, I., Darwich, L., Casal, J., Martin, M. and Pujols, J. (2006) Evolution of ORF5 of Spanish porcine reproductive and respiratory syndrome virus strains from 1991 to 2005. Virus Res 115(2), 198-206.

Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R., Schnitzlein, W. and Zuckermann, F.A. (2003) Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309(1), 18-31.

Meng, X.J. (2000) Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet Microbiol 74(4), 309-29.

Meng, X.J., Paul, P.S. and Halbur, P.G. (1994) Molecular-Cloning and Nucleotide Sequencing of the 3'-Terminal Genomic Rna of the Porcine Reproductive and Respiratory Syndrome Virus. Journal of General Virology 75, 1795-1801.

Meng, X.J., Paul, P.S., Halbur, P.G. and Lum, M.A. (1995a) Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch Virol 140(4), 745-55.

Meng, X.J., Paul, P.S., Halbur, P.G. and Lum, M.A. (1996) Characterization of a high-virulence US isolate of porcine reproductive and respiratory syndrome virus in a continuous cell line, ATCC CRL11171. Journal of Veterinary Diagnostic Investigation 8(3), 374-381.

Meng, X.J., Paul, P.S., Halbur, P.G. and Morozov, I. (1995b) Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. J Gen Virol 76 (Pt 12), 3181-8.

Meulenberg, J.J. and Petersen-den Besten, A. (1996) Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP2 encoded by ORF2 is incorporated in virus particles. Virology 225(1), 44-51.

Meulenberg, J.J., van Nieuwstadt, A.P., van Essen-Zandbergen, A. and Langeveld, J.P. (1997) Posttranslational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. J Virol 71(8), 6061-7.

Meulenberg, J.J.M. (2000) PRRSV, the virus. Veterinary Research 31(1), 11-21.

Meulenberg, J.J.M., BosDeRuijter, J.N.A., vandeGraaf, R., Wensvoort, G. and Moormann, R.J.M. (1998) Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. Journal of Virology 72(1), 380-387.

Meulenberg, J.J.M., Petersendenbesten, A., Dekluyver, E.P., Moormann, R.J.M., Schaaper, W.M.M. and Wensvoort, G. (1995) Characterization of Proteins Encoded by Orf-2 to Orf-7 of Lelystad-Virus. Virology 206(1), 155-163.

Miller, L.C., Laegreid, W.W., Bono, J.L., Chitko-McKown, C.G. and Fox, J.M. (2004) Interferon type I response in porcine reproductive and respiratory syndrome virus-infected MARC-145 cells. Arch Virol 149(12), 2453-63.

Morozov, I., Meng, X.J. and Paul, P.S. (1995) Sequence analysis of open reading frames (ORFs) 2 to 4 of a U.S. isolate of porcine reproductive and respiratory syndrome virus. Arch Virol 140(7), 1313-9.

Murtaugh, M.P., Elam, M.R. and Kakach, L.T. (1995) Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. Arch Virol 140(8), 1451-60.

Murtaugh, M.P., Xiao, Z.G. and Zuckermann, F. (2002) Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunology 15(4), 533-547.

Nauwynck, H.J., Duan, X., Favoreel, H.W., Van Oostveldt, P. and Pensaert, M.B. (1999) Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis. Journal of General Virology 80, 297-305.

Nedialkova, D.D., Ulferts, R., van den Born, E., Lauber, C., Gorbalenya, A.E., Ziebuhr, J. and Snijder, E.J. (2009) Biochemical Characterization of Arterivirus Nonstructural Protein 11 Reveals the Nidovirus-Wide Conservation of a Replicative Endoribonuclease. Journal of Virology 83(11), 5671-5682.

Nelsen, C.J., Murtaugh, M.P. and Faaberg, K.S. (1999) Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J Virol 73(1), 270-80.

Nelson, E.A., Christopher-Hennings, J. and Benfield, D.A. (1994) Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. J Vet Diagn Invest 6(4), 410-5.

Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E. and Benfield, D.A. (1993) Differentiation of U.S. and European isolates of porcine

reproductive and respiratory syndrome virus by monoclonal antibodies. J Clin Microbiol 31(12), 3184-9.

Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L. and Zimmerman, J.J. (2005) Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J Am Vet Med Assoc 227(3), 385-92.

Nielsen, H.S., Liu, G., Nielsen, J., Oleksiewicz, M.B., Botner, A., Storgaard, T. and Faaberg, K.S. (2003) Generation of an infectious clone of VR-2332, a highly virulent North American type isolate of porcine reproductive and respiratory syndrome virus. Journal of Virology 77(6), 3702-3711.

Nielsen, H.S., Oleksiewicz, M.B., Forsberg, R., Stadejek, T., Botner, A. and Storgaard, T. (2001) Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. J Gen Virol 82(Pt 6), 1263-72.

Nielsen, J., Botner, A., Bille-Hansen, V., Oleksiewicz, M.B. and Storgaard, T. (2002) Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus. Vet Microbiol 84(1-2), 1-13.

Nielsen, T.L., Nielsen, J., Have, P., Baekbo, P., Hoff-Jorgensen, R. and Botner, A. (1997) Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. Vet Microbiol 54(2), 101-12.

Okuda, Y., Kuroda, M., Ono, M., Chikata, S. and Shibata, I. (2008) Efficacy of vaccination with porcine reproductive and respiratory syndrome virus following challenges with field isolates in Japan. J Vet Med Sci 70(10), 1017-25.

Osorio, F.A., Galeota, J.A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F. and Laegreid, W.W. (2002) Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. Virology 302(1), 9-20.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A. and Lopez, O.J. (2002) Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J Virol 76(9), 4241-50.

Parker, K.C., Bednarek, M.A. and Coligan, J.E. (1994) Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 152(1), 163-75.

Pirzadeh, B. and Dea, S. (1997) Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. J Gen Virol 78 (Pt 8), 1867-73.

Plagemann, P.G. (2004) The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 102(3), 263-75.

Plagemann, P.G. (2006) Neutralizing antibody formation in swine infected with seven strains of porcine reproductive and respiratory syndrome virus as measured by indirect ELISA with peptides containing the GP5 neutralization epitope. Viral Immunol 19(2), 285-93.

Plagemann, P.G., Rowland, R.R. and Faaberg, K.S. (2002) The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch Virol 147(12), 2327-47.

Plana, J., Vayreda, M., Vilarrasa, J., Bastons, M., Rosell, R., Martinez, M., Gabriel, A.S., Pujols, J., Badiola, J.L., Ramos, J.A. and Domingo, M. (1992) Porcine Epidemic Abortion and Respiratory Syndrome (Mystery Swine Disease) - Isolation in Spain of the Causative Agent and Experimental Reproduction of the Disease. Veterinary Microbiology 33(1-4), 203-211.

Prieto, C., Alvarez, E., Martinez-Lobo, F.J., Simarro, I. and Castro, J.M. (2008) Similarity of European porcine reproductive and respiratory syndrome virus strains to vaccine strain is not necessarily predictive of the degree of protective immunity conferred. Vet J 175(3), 356-63.

Prieto, C., Vazquez, A., Nunez, J.I., Alvarez, E., Simarro, I. and Castro, J.M. (2009) Influence of time on the genetic heterogeneity of Spanish porcine reproductive and respiratory syndrome virus isolates. Vet J 180(3), 363-70.

Qiu, H.J., Tian, Z.J., Tong, G.Z., Zhou, Y.J., Ni, J.Q., Luo, Y.Z. and Cai, X.H. (2005) Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. Vet Immunol Immunopathol 106(3-4), 309-19.

Rammensee, H., Bachmann, J., Emmerich, N.P., Bachor, O.A. and Stevanovic, S. (1999) SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50(3-4), 213-9.

Riddell, S.R., Reusser, P. and Greenberg, P.D. (1991) Cytotoxic T cells specific for cytomegalovirus: a potential therapy for immunocompromised patients. Rev Infect Dis 13 Suppl 11, S966-73.

Riddell, S.R., Watanabe, K.S., Goodrich, J.M., Li, C.R., Agha, M.E. and Greenberg, P.D. (1992) Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 257(5067), 238-41.

Ropp, S.L., Wees, C.E., Fang, Y., Nelson, E.A., Rossow, K.D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J.E., Benfield, D.A. and Faaberg, K.S. (2004) Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. J Virol 78(7), 3684-703.

Rossow, K.D. (1998) Porcine reproductive and respiratory syndrome. Veterinary Pathology 35(1), 1-20.

Rossow, K.D., Bautista, E.M., Goyal, S.M., Molitor, T.W., Murtaugh, M.P., Morrison, R.B., Benfield, D.A. and Collins, J.E. (1994) Experimental Porcine Reproductive and Respiratory Syndrome Virus-Infection in One-Week-Old, 4-Week-Old, and 10-Week-Old Pigs. Journal of Veterinary Diagnostic Investigation 6(1), 3-12.

Rossow, K.D., Collins, J.E., Goyal, S.M., Nelson, E.A., Christopherhennings, J. and Benfield, D.A. (1995) Pathogenesis of Porcine Reproductive and Respiratory Syndrome Virus-Infection in Gnotobiotic Pigs. Veterinary Pathology 32(4), 361-373.

Rowland, R.R., Steffen, M., Ackerman, T. and Benfield, D.A. (1999) The evolution of porcine reproductive and respiratory syndrome virus: quasispecies and emergence

of a virus subpopulation during infection of pigs with VR-2332. Virology 259(2), 262-6.

Schneider, T.D. and Stephens, R.M. (1990) Sequence logos: a new way to display consensus sequences. Nucleic Acids Res 18(20), 6097-100.

Scortti, M., Prieto, C., Alvarez, E., Simarro, I. and Castro, J.M. (2007) Failure of an inactivated vaccine against porcine reproductive and respiratory syndrome to protect gilts against a heterologous challenge with PRRSV. Vet Rec 161(24), 809-13.

Seth, R.B., Sun, L. and Chen, Z.J. (2006) Antiviral innate immunity pathways. Cell Res 16(2), 141-7.

Singh, H. and Raghava, G.P. (2001) ProPred: prediction of HLA-DR binding sites. Bioinformatics 17(12), 1236-7.

Snijder, E.J. and Meulenberg, J.J.M. (1998) The molecular biology of arteriviruses. Journal of General Virology 79, 961-979.

Storgaard, T., Oleksiewicz, M. and Botner, A. (1999) Examination of the selective pressures on a live PRRS vaccine virus. Arch Virol 144(12), 2389-401.

Streeck, H., Frahm, N. and Walker, B.D. (2009) The role of IFN-gamma Elispot assay in HIV vaccine research. Nat Protoc 4(4), 461-9.

Terpstra, C., Wensvoort, G. and Pol, J.M.A. (1991) Experimental Reproduction of Porcine Epidemic Abortion and Respiratory Syndrome (Mystery Swine Disease) by Infection with Lelystad Virus - Koch Postulates Fulfilled. Veterinary Quarterly 13(3), 131-136.

Truong, H.M., Lu, Z., Kutish, G.F., Galeota, J., Osorio, F.A. and Pattnaik, A.K. (2004) A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus. Virology 325(2), 308-19.

Van Gorp, H., Van Breedam, W., Delputte, P.L. and Nauwynck, H.J. (2008) Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. J Gen Virol 89(Pt 12), 2943-53.

Vanderheijden, N., Delputte, P.L., Favoreel, H.W., Vandekerckhove, J., Van Damme, J., van Woensel, P.A. and Nauwynck, H.J. (2003) Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. J Virol 77(15), 8207-15.

Vanhee, M., Costers, S., Van Breedam, W., Geldhof, M.F., Van Doorsselaere, J. and Nauwynck, H.J. A variable region in GP4 of European-type porcine reproductive and respiratory syndrome virus induces neutralizing antibodies against homologous but not heterologous virus strains. Viral Immunol 23(4), 403-13.

Vashisht, K., Goldberg, T.L., Husmann, R.J., Schnitzlein, W. and Zuckermann, F.A. (2008) Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus. Vaccine 26(36), 4747-53.

Wang, X., Eaton, M., Mayer, M., Li, H., He, D., Nelson, E. and Christopher-Hennings, J. (2007) Porcine reproductive and respiratory syndrome virus productively infects monocyte-derived dendritic cells and compromises their antigen-presenting ability. Arch Virol 152(2), 289-303.

Wang, Y.X., Zhou, Y.J., Li, G.X., Zhang, S.R., Jiang, Y.F., Xu, A.T., Yu, H., Wang, M.M., Yan, L.P. and Tong, G.Z. (2011) Identification of immunodominant T-cell epitopes in membrane protein of highly pathogenic porcine reproductive and respiratory syndrome virus. Virus Res 158(1-2), 108-15.

Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M. and Barton, G.J. (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics 25(9), 1189-91.

Weiland, E., Wieczorek-Krohmer, M., Kohl, D., Conzelmann, K.K. and Weiland, F. (1999) Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. Vet Microbiol 66(3), 171-86.

Wensvoort, G. (1993) Lelystad Virus and the Porcine Epidemic Abortion and Respiratory Syndrome. Veterinary Research 24(2), 117-124.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. and et al. (1991) Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet Q 13(3), 121-30.

Wesley, R.D., Lager, K.M. and Kehrli, M.E., Jr. (2006) Infection with Porcine reproductive and respiratory syndrome virus stimulates an early gamma interferon response in the serum of pigs. Can J Vet Res 70(3), 176-82.

Wieczorek-Krohmer, M., Weiland, F., Conzelmann, K., Kohl, D., Visser, N., van Woensel, P., Thiel, H.J. and Weiland, E. (1996) Porcine reproductive and respiratory syndrome virus (PRRSV): monoclonal antibodies detect common epitopes on two viral proteins of European and U.S. isolates. Vet Microbiol 51(3-4), 257-66.

Wills, R.W., Zimmerman, J.J., Yoon, K.J., Swenson, S.L., McGinley, M.J., Hill, H.T., Platt, K.B., Christopher-Hennings, J. and Nelson, E.A. (1997) Porcine reproductive and respiratory syndrome virus: a persistent infection. Vet Microbiol 55(1-4), 231-40.

Wissink, E.H.J., Kroese, M.V., van Wijk, H.A.R., Rijsewijk, F.A.M., Meulenberg, J.J.M. and Rottier, P.J.M. (2005) Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. Journal of Virology 79(19), 12495-12506.

Wu, W.H., Fang, Y., Farwell, R., Steffen-Bien, M., Rowland, R.R.R., Christopher-Hennings, J. and Nelson, E.A. (2001) A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. Virology 287(1), 183-191.

Wulf, M., Hoehn, P. and Trinder, P. (2009) Identification and validation of T-cell epitopes using the IFN-gamma ELISPOT assay. Methods in molecular biology 524, 439-46.

Xiao, Z., Batista, L., Dee, S., Halbur, P. and Murtaugh, M.P. (2004) The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. J Virol 78(11), 5923-33.

Yang, L., Frey, M.L., Yoon, K.J., Zimmerman, J.J. and Platt, K.B. (2000) Categorization of North American porcine reproductive and respiratory syndrome viruses:

epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. Arch Virol 145(8), 1599-619.

Yang, O.O., Nguyen, P.T., Kalams, S.A., Dorfman, T., Gottlinger, H.G., Stewart, S., Chen, I.S., Threlkeld, S. and Walker, B.D. (2002) Nef-mediated resistance of human immunodeficiency virus type 1 to antiviral cytotoxic T lymphocytes. J Virol 76(4), 1626-31.

Yoon, I.J., Joo, H.S., Goyal, S.M. and Molitor, T.W. (1994) A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera. J Vet Diagn Invest 6(3), 289-92.

Yoon, K.J., Wu, L.L., Zimmerman, J.J., Hill, H.T. and Platt, K.B. (1996) Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Viral Immunol 9(1), 51-63.

Yoon, K.J., Zimmerman, J.J., Swenson, S.L., McGinley, M.J., Eernisse, K.A., Brevik, A., Rhinehart, L.L., Frey, M.L., Hill, H.T. and Platt, K.B. (1995) Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. J Vet Diagn Invest 7(3), 305-12.

Yuan, S., Nelsen, C.J., Murtaugh, M.P., Schmitt, B.J. and Faaberg, K.S. (1999) Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus Res 61(1), 87-98.

Zuckermann, F.A., Garcia, E.A., Luque, I.D., Christopher-Hennings, J., Doster, A., Brito, M. and Osorio, F. (2007) Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Vet Microbiol 123(1-3), 69-85.