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MAPEAMENTO E DELEÇÃO DE EPÍTOPOS LINEARES DE LINFÓCITOS B EM PROTEÍNAS DO VÍRUS DA SÍNDROME RESPIRATÓRIA E REPRODUTIVA DOS SUÍNOS PARA A PRODUÇÃO DE UMA VACINA DIFERENCIAL

TESE DE DOUTORADO

Marcelo de Lima

Santa Maria, RS, Brasil
2008

MAPEAMENTO E DELEÇÃO DE EPÍTOPOS LINEARES DE LINFÓCITOS B EM PROTEÍNAS DO VÍRUS DA SÍNDROME RESPIRATÓRIA E REPRODUTIVA DOS SUÍNOS PARA A PRODUÇÃO DE UMA VACINA DIFERENCIAL

Por

Marcelo de Lima

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária.

Orientador: Prof. Eduardo Furtado Flores

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Universidade Federal de Santa Maria
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Programa de Pós-Graduação em Medicina Veterinária
Departamento de Medicina Veterinária Preventiva

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Elaborada por
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Como requisito parcial para obtenção do grau de
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RESUMO
Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

MAPEAMENTO E DELEÇÃO DE EPÍTOPOS LINEARES DE LINFÓCITOS B EM PROTEÍNAS DO VÍRUS DA SÍNDROME RESPIRATÓRIA E REPRODUTIVA DOS SUÍNOS PARA A PRODUÇÃO DE UMA VACINA DIFERENCIAL
AUTOR: MARCELO DE LIMA
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Santa Maria, 25 de fevereiro de 2008

O vírus da síndrome respiratória e reprodutiva dos suínos (PRRSV) foi isolado pela primeira vez em 1991 e, desde então, tem sido associado a perdas significativas para a suinocultura mundial. Apesar da vacinação contra o PRRSV ser amplamente utilizada, um grande avanço seria alcançado com a elaboração de vacinas diferenciais que permitam a discriminação sorológica entre animais vacinados e naturalmente infectados. O presente estudo teve como objetivo a identificação de regiões imunogênicas, conservadas e dispensáveis a replicação viral, em diferentes proteínas do PRRSV, que pudessem ser utilizadas como marcadores sorológicos negativos em uma nova geração de vacinas atenuadas. Na primeira parte desta tese estão apresentados os resultados de um mapeamento de epítopos lineares de linfócitos B em diferentes proteínas do PRRSV, pelo uso da tecnologia de *Pepscan*. Os resultados indicam a presença de diversas regiões imunodominantes na proteína não estrutural 2 (Nsp2) e em todas as proteínas estruturais do vírus. Essas regiões foram consistentemente reconhecidas pelo soro de suínos experimentalmente infectados com uma cepa norte-americana do PRRSV (NVSL97-7895). A maior freqüência de epítopos imunodominantes foi identificada na Nsp2 (n=18) e o mais alto grau de imunogenicidade e nível de conservação de aminoácidos foi observado em dois epítopos identificados na extremidade carboxi-terminal da proteína M (ORF6). Anticorpos reagentes com epítopos imunodominantes de cada proteína foram detectados inicialmente entre os dias 7-15 pós-infecção (pi), permanecendo em altos títulos até o final do experimento (dia 90 pi). Com base na imunodominância e nível de conservação de amino ácidos (aa) das seqüências mapeadas, dois epítopos alvos foram selecionados como candidatos a marcadores sorológicos negativos em cada uma das proteínas Nsp2, Gp3 e M. Esses epítopos foram então deletados em um clone infeccioso de cDNA (FL12) por mutagênese sítio-direcionada. Os resultados desses experimentos encontram-se descritos na segunda parte da tese. Um vírus

mutante carreando a deleção de um epítopo imunodominante da Nsp2 (FLdNsp2/44) foi obtido após transfeccção de RNA viral em células MARC145. A caracterização *in vitro* e *in vivo* do vírus mutante demonstrou que a remoção dos 15 aa da Nsp2 não produziu efeito sobre a imunogenicidade, replicação ou virulência quando comparado ao vírus parental. Além disso, observou-se indução de soroconversão contra o PRRSV em animais infectados, detectada pelo uso de um teste ELISA comercial. Por outro lado, não foi detectada resposta humoral específica contra a região deletada nos animais imunizados com o FLdNsp2/44, conforme resultados de um teste ELISA contendo como antígeno um peptídeo sintético correspondente a seqüência removida. Por outro lado, deleções dos epítopos previamente identificados na Gp3 e proteína M foram letais à viabilidade viral *in vitro*. Alternativamente, um outro vírus mutante foi gerado pela substituição de 5 aa do epítopo identificado na proteína M, embora a alteração de resíduos não tenha sido suficiente para eliminar a imunogenicidade da região. Em resumo, os resultados do presente estudo se constituem em uma prova de conceito no sentido do desenvolvimento de vacinas diferenciais contra o PRRSV. A utilização de um vírus mutante carreando a deleção de um epítopo imunodominante, associado com um teste de ELISA baseado no peptídeo sintético correspondente a região deletada, representam uma alternativa para o desenvolvimento de vacinas diferenciais atenuadas contra o PRRSV.

Palavras-chave: PRRSV, epítopos lineares de células B, peptídeos, *Pepscan,* clone infeccioso, vacina diferencial.

ABSTRACT
Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

MAPPING AND DELETION OF B-CELL LINEAR EPITOPES IN PROTEINS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS FOR THE PRODUCTION OF A DIFFERENTIAL VACCINE
AUTHOR: MARCELO DE LIMA
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Santa Maria, February 25th, 2008

Porcine reproductive and respiratory syndrome virus (PRRSV) was isolated for the first time in 1991 and since then it has been associated with significant economic losses to the pig industry worldwide. Although vaccination against PRRSV is widely used, an important advance would be the development of marker vaccines allowing serologic discrimination between vaccinated and naturally infected animals. The present study aimed to identify immunogenic and conserved regions dispensable to viral replication in different PRRSV proteins, which could be used as negative serologic markers in a new generation of live-attenuated vaccines. A fine mapping of B-cell linear epitopes in different PRRSV proteins by Pepscan is presented in the first part of this thesis. The results indicated the presence of several B-cell linear epitopes in the non-structural protein 2 (Nsp2) and in all structural proteins encoded by PRRSV, which were consistently recognized by antibodies raised in pigs experimentally infected with a North American strain of the virus (NVSL97-7895). The Nsp2 was found to harbor the highest frequency of immunodominant epitopes (n=18) when compared to structural proteins. In the structural proteins, epitopes consistently recognized by immune sera were located in all studied proteins. Overall, the highest degree of immunogenicity and conservation was exhibited by two epitopes identified in the C-terminal end of the M protein (ORF6). The antibodies recognizing the immunodominant epitopes of each protein were detected as early as days 7 to 15 post-infection (p.i.) and remained detectable until the end of the experiment (day 90 p.i). Based on their immunodominance and level of amino acid (aa) conservation, two target epitopes were selected to serve as serological marker candidates in each of the following PRRSV proteins: Nsp2, GP3 and M. These epitopes were deleted in the wild-type cDNA infectious clone (FL-12) by site-directed mutagenesis. The results of this study are presented in the second part of this thesis. A Nsp2 mutant virus (FLdNsp2/44) was successfully rescued following RNA transfection in MARC

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Keywords: PRRSV, B-cell linear epitopes, peptides, Pepscan, infectious cDNA clone, site-directed mutagenesis, marker vaccines.

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1. INTRODUÇÃO

A síndrome respiratória e reprodutiva dos suínos (PRRS) é uma enfermidade de grande importância para a suinocultura mundial devido às perdas econômicas decorrentes da infecção em animais suscetíveis. A doença foi inicialmente observada em granjas de suínos de diferentes estados norte-americanos no final da década de 80. Curiosamente, surtos com características clínicas muito similares foram também relatados na Europa e Ásia no início da década de 90 (ZIMMERMAN, 2003a). Apesar de estudos sorológicos retrospectivos indicarem a circulação do agente etiológico vários anos antes da doença tornar-se conhecida, a etiologia viral foi somente definida em 1991, pelo isolamento de um vírus RNA por pesquisadores do Instituto Central de Veterinária de Lelystad, na Holanda (WENSVOORT et al., 1991). Desde então o vírus ficou amplamente conhecido como vírus da síndrome respiratória e reprodutiva dos suínos (PRRSV).

Estudos de caracterização do PRRSV demonstraram semelhanças na estrutura e morfologia dos vírions, na seqüência de nucleotídeos e organização genômica, além de propriedades biológicas em comum do vírus da arterite eqüina (EAV), vírus elevador da lactato-desidrogenase (LDEV) e do vírus da febre hemorrágica dos símios (SHFV). Essas características similares levaram estes agentes a serem agrupados em uma nova família viral, a *Arteriviridae.*

Apesar da infecção pelo PRRSV ser considerada endêmica na maioria dos países que possuem a suinocultura expressiva (HOLCK & POLSON, 2003; ZIMMERMANN, 2003b), o Brasil é considerado área livre (CIACCI-ZANELLA et al., 2004). Entretanto, é essencial ressaltar a importância de um monitoramento constante do rebanho suíno brasileiro, principalmente, em relação à introdução de animais e material genético no país.

Apesar dos esforços direcionados ao controle e profilaxia das infecções causadas pelo PRRSV desde a sua identificação, o vírus continua ainda a causar prejuízos significativos em países produtores de suínos (NEUMANN et al., 2005). A dificuldade na obtenção de vacinas mais eficazes e seguras demonstra claramente que muitos aspectos relacionados com a biologia dos arterivírus não estão completamente elucidados.

Vacinas atenuadas e inativadas contra o PRRSV estão comercialmente disponíveis, sendo amplamente utilizadas nos Estados Unidos e Europa. Porém, entre outros aspectos, é discutível a eficácia das vacinas atuais, demonstrando a necessidade de uma reformulação desses imunógenos. Dentre as características desejáveis nessa nova geração de vacinas, estaria a identificação de marcadores sorológicos negativos, ou seja, regiões imunogênicas e

conservadas de diferentes proteínas virais dispensáveis a replicação viral. A identificação de marcadoes sorológicos associado ao desenvolvimento de um teste sorológico diferencial, permitiria a diferenciação entre animais vacinados e infectados naturalmente, um aspecto que contribuiria para eventuais programas de erradicação.

A obtenção de clones infecciosos pelo uso da genética reversa representou um grande avanço para o estudo do PRRSV em nível molecular (TRUONG et al., 2004; KWON et al., 2006; YOO et al., 2004; MEULENBERG et al., 1998; NIELSEN et al., 2003). Essa tecnologia permite modificações pré-definidas no genoma viral (deleções, inserções e/ou substituições de nucleotídeos), possibilitando estudos detalhados dos mecanismos moleculares relacionados com patogenia, replicação, persistência e imunidade. Com isso, é possível a manipulação genômica visando o desenvolvimento de cepas vacinais geneticamente atenuadas, ou com alterações em proteínas virais que possam ser utilizadas como marcadores sorológicos (YOO et al., 2004). Tais modificações são desejáveis e essenciais para o desenvolvimento de uma nova geração de vacinas atenuadas para serem utilizadas na profilaxia, controle e eventual erradicação do PRRSV.

O presente trabalho descreve um mapeamento detalhado de epítopos lineares de linfócitos B em várias proteínas do PRRSV (Capítulo 2). Dentre as regiões identificadas, diversos segmentos imunodominantes e conservados foram selecionados como candidatos a marcadores sorológicos negativos. Posteriormente, foi investigada a dispensabilidade destas regiões para a replicação viral, bem como o seu efeito na imunogenicidade e virulência dos vírus recombinantes em animais (Capítulo 3).

2. CAPÍTULO 1

REVISÃO DE LITERATURA

No final da década de 80, surtos de uma doença até então desconhecida foram relatados simultaneamente em granjas de suínos nos estados da Carolina do Norte, Indiana, Minnesota e Iowa, nos Estados Unidos. A síndrome consistia em perdas reprodutivas, pneumonia pós-desmame em leitões, retardo no crescimento e aumento na taxa de mortalidade (HILL, 1990). Surtos semelhantes foram também observados na Europa e Ásia no início da década de 90 (ZIMMERMAN, 2003a). A enfermidade foi inicialmente denominada de Doença Misteriosa dos Suínos e Síndrome Respiratória e Infertilidade Suína (GOYAL, 1993), devido a dificuldade na identificação do agente envolvido. A etiologia viral foi finalmente definida em 1991 (WENSVOORT et al., 1991) e a doença ficou posteriormente conhecida como Síndrome Reprodutiva e Respiratória dos Suínos (PRRS).

Atualmente, acredita-se que a infecção pelo PRRSV seja endêmica na maioria dos países produtores de suínos, causando um impacto econômico significativo na suinocultura mundial (HOLCK & POLSON, 2003; ZIMMERMANN, 2003b). Somente nos Estados Unidos, estima-se que a infecção pelo PRRSV resulte em prejuízos anuais superiores a 560 milhões de dólares à indústria de suínos (NEUMANN et al., 2005). MALDONADO et al., (2005) demonstraram que o PRRSV foi o agente viral mais importante associado com perdas reprodutivas e infecção fetal em suínos na Espanha. Além das perdas na produção, os custos relacionados com a prevenção de infecções secundárias e com a saúde geral dos rebanhos são muito superiores quando comparados ao período pré-infecção (HOLCK & POLSON, 2003). Uma forma atípica da PRRS com altas taxas de mortalidade foi identificada na China em 2006, onde cepas altamente virulentas do vírus foram isoladas de animais infectados (TIAN et al., 2007; LI et al., 2007).

No Chile, o governo estabeleceu um programa de erradicação do PRRSV conduzido pelo *Servicio Agricola Ganadero* (SAG) em colaboração com a indústria suinícola representada pela Associação Chilena de Produtores de Suínos (ASPROCER). O programa encontra-se em fases avançadas, sendo que a grande maioria das granjas já são consideradas livres da infecção e serão mantidas negativas por um monitoramento contínuo e certificação através de sorologia (Dr. Leonardo Cuevas, ASPROCER – comunicação pessoal, 2007).

Apesar de sua ampla distribuição, alguns países europeus (Suécia, Suíça, Noruega, Finlândia), além de Nova Zelândia, Austrália, Brasil, Argentina e algumas áreas do Caribe são

consideradas livres da PRRS. No Brasil, a ausência da infecção foi demonstrada pela realização de estudos sorológicos e virológicos em granjas de suínos de diferentes estados (CIACCI-ZANELLA et al., 2004). No entanto, tendo em vista a importância da suinocultura brasileira no agronegócio nacional e internacional, é indispensável um monitoramento contínuo dos rebanhos, assim como de animais e material genético introduzido no país.

O PRRSV está classificado na ordem *Nidovirales*, família *Arteriviridae*, gênero *Arterivirus*, juntamente com o vírus da arterite viral eqüina (EAV), *lactate dehydrogenase-elevating virus* (LDV) e o vírus da febre hemorrágica dos símios (SHFV). É um vírus pequeno (50-65nm de diâmetro), com nucleocapsídeo possivelmente icosaédrico com diâmetro entre 25-35nm, envolto por um envelope lipoprotéico. O genoma consiste de uma molécula linear de RNA, fita simples e sentido positivo, com aproximadamente 15.1Kb. (SNIJDER & MEULENBERG, 2001).

Após o seqüenciamento completo do genoma de cepas européias (MEULENBERG et al., 1993) e norte-americanas (ALLENDE et al., 1999), observou-se que a organização genômica do PRRSV é muito similar a de outros arterivirus. Basicamente, o genoma contém 8 ORFs *(open reading frames)*. As ORFs 1a e 1b abrangem cerca de 80% do genoma viral. A ORF1a codifica uma poliproteína que é posteriormente clivada, originando 8 polipeptídeos (Nsp1 a Nsp8). Essas proteínas possuem atividade proteolítica, sendo responsáveis pelo processamento de outras Nsps. A clivagem proteolítica da ORF1b gera os polipeptídeos Nsp9 a Nsp12. As regiões com atividade de RNA polimerase e NTPase/RNA helicase estão associadas com a Nsp9 e Nsp10, respectivamente. As outras seis ORFs (2 a 7), localizadas na extremidade 3' do genoma, codificam proteínas estruturais que permanecem como componentes do vírion. As ORFs 2 a 7 são expressas a partir de um grupo de RNA mensageiros subgenômicos (RNAsg) compostos de uma sequência *leader* derivada da extremidade 5' do genoma viral e fusionada ao RNA mensageiro através de um mecanismo de transcrição descontínua (MEULENBERG, 2000).

As três principais proteínas estruturais (gp5, M e N) são codificadas a partir de RNAs mensageiros subgenômicos transcritos a partir da extremidade 3' do genoma do PRRSV. A proteína N (ORF7) é pequena (12-15KDa) e constitui 20 a 40% da massa protéica do vírion. A proteína M (ORF6) é uma proteína de membrana, não glicosilada, sendo a mais conservada proteína estrutural dos arterivirus. Acumula-se no retículo endoplasmático de células infectadas, onde interage com a principal glicoproteína do envelope viral (gp5) formando heterodímeros que são essenciais para a infectividade viral (SNIJDER & MEULENBERG, 2001). Os produtos das ORFs 2 a 4 provavelmente possuem uma importância menor e a exata

função dessas proteínas ainda não foi completamente elucidada. WU et al. (2001) demonstraram a presença de uma ORF adicional dentro da ORF2 (ORF2b), que codifica uma proteína estrutural não glicosilada (E) de 10kDa. Entretanto, algumas cepas norte-americanas não contêm o códon de iniciação da ORF2b (WU et al., 2001). Essa mesma ORF adicional também já foi identificada em cepas européias do PRRSV (SNIJDER et al., 1999).

A verdadeira origem do PRRSV ainda permanece indefinida. Evidências indicam que o PRRSV já infectava suínos domésticos vários anos antes da doença tornar-se conhecida e economicamente importante. Um estudo sorológico retrospectivo em amostras de soro coletadas de suínos no final da década de 70 e nos anos 80 no Canadá, Coréia, Japão e Alemanha, demonstrou a presença de anticorpos específicos contra o PRRSV (BLAHA et al., 2000). HANADA et al. (2005) sugerem que o PRRSV foi transferido de um outro hospedeiro para suínos, aproximadamente no início da década de 80, e sofreu adaptação a células suínas através da alteração de regiões transmembrana da ORF5. Utilizando uma metodologia alternativa, FORSBERG (2005) concluiu que o ancestral comum mais recente de todos os isolados de PRRSV existiu ao redor de 1880, aproximadamente 100 anos antes da data estimada por HANADA et al. (2005).

PLAGEMANN (2003) sugeriu que o PRRSV originou-se a partir do LDV e que suínos selvagens na Europa serviram como hospedeiros intermediários antes do vírus adquirir a capacidade de infectar suínos domésticos. Assim, o vírus teria sido transferido para a América do Norte pela importação desses animais em 1912. Essa hipótese poderia explicar o longo período de evolução independente nos dois continentes e estaria de acordo com o momento de divergência a partir de um ancestral comum estimado ao redor de 1880 (FORSBERG, 2005). Uma análise comparativa entre as cepas protótipo do tipo norte-americano do PRRSV (VR2332) e o Europeu (*Lelystad virus* - LV) revelou diferenças substanciais em nível genômico e também no processamento dos RNAs mensageiros subgenômicos, indicando que o PRRSV evoluiu independentemente nos dois continentes (NELSEN et al., 1999). Entretando, apesar de diversos estudos procurando elucidar a origem do PRRSV, ainda não existem explicações satisfatórias demonstrando como os dois subtipos emergiram quase simultaneamente na Europa e América do Norte.

PESCH et al. (2005) monitorando a variação genética entre isolados europeus desde 1991, observaram que a distância genética entre esses isolados tem aumentado com o passar do tempo. Também já foi demonstrado que o PRRSV possui uma taxa de evolução mais rápida do que outros vírus RNA (HANADA et al., 2005).

O período após a exposição de animais suscetíveis ao PRRSV é caracterizado, em muitos casos, pela abundante replicação viral em macrófagos alveolares e teciduais. Em fases tardias é freqüente a ocorrência de persistência viral, caracterizada por baixos níveis de replicação, primariamente em tecidos linfóides. Eventualmente, o vírus é completamente eliminado pelo sistema imunológico dos animais com infecção persistente, sendo que na maioria dos casos esse período pode levar vários meses (ALLENDE et al., 2000).

Embora os mecanismos responsáveis pela persistência do PRRSV ainda não estejam completamente elucidados, a incapacidade do sistema imunológico do hospedeiro em desenvolver uma resposta imune efetiva contra o vírus parece ser um dos principais fatores relacionados com a persistência viral em animais convalescentes (LOPEZ & OSORIO, 2004). Além disso, um retardo na produção de interferon gama (MEIER et al., 2003), bem como na indução de anticorpos neutralizantes têm sido observado (OSTROWSKI et al., 2002), constituindo-se em mecanismos potenciais de evasão do sistema imunológico.

Estudos com anticorpos monoclonais e policlonais revelaram diferenças antigênicas importantes entre cepas norte-americanas e européias e também entre isolados norte-americanos do PRRSV (NELSON et al., 1993). A análise de cepas de referência isoladas nos Estados Unidos e Europa demonstraram que a identidade de aminoácidos entre as seqüências analisadas é inferior a 60% (WENSVOORT et al., 1992; ALLENDE et al., 1999). Com base nessas diferenças, os isolados de PRRSV foram divididos em dois genótipos: tipo I (Europeu) e tipo II (Norte Americano). De um modo geral, os isolados do genótipo I são restritos ao continente Europeu enquanto que os isolados do genótipo II são encontrados nos Estados Unidos, Canadá, México e também em países asiáticos. Entretanto, isolados do genótipo II já foram identificados na Europa, apresentando um alto grau de homologia com uma vacina atenuada Norte Americana introduzida no continente em 1995. Por outro lado, isolados do tipo I também já foram identificados nos Estados Unidos, porém a origem exata ainda não foi determinada (FANG et al., 2004).

Entre as medidas utilizadas para a prevenção e controle das infecções causadas pelo PRRSV, está o uso da vacinação com cepas atenuadas (GILLESPIE, 2003) ou formulações inativadas do vírus (THACKER et al., 2003). Nos Estados Unidos, a vacinação contra a PRRS tem sido realizada desde 1995, sendo que a vacina comercial comumente utilizada contém uma cepa virulenta do vírus atenuada por múltiplas passagens em cultivo celular. Estudos têm demonstrado que essas vacinas induzem imunidade protetora contra o vírus homólogo, mas produzem níveis variáveis de proteção heteróloga (MENGELING et al., 1996).

OSORIO et al. (1998) avaliaram a eficácia de duas vacinas atenuadas e uma vacina autógena inativada através da imunização de fêmeas prenhes e posterior desafio com amostras virulentas do PRRSV. Nesse estudo, foi demonstrada uma viabilidade entre 50-60% ao desmame de leitões nascidos de fêmeas imunizadas com as vacinas atenuadas, e de apenas 10% dos leitões provenientes de fêmeas que receberam a vacina inativada. Falha na prevenção de sinais clínicos, viremia e infecção transplacentária após o desafio com amostras virulentas do vírus, também foram observados em animais imunizados com uma vacina contendo uma cepa Européia do PRRSV (SCORTTI et al., 2007).

Além da eficácia discutida das atuais vacinas disponíveis para a prevenção e controle das infecções causadas pelo PRRSV, outro problema está relacionado com a segurança das vacinas atenuadas. Persistência do vírus vacinal em animais imunizados, com um padrão similar ao de amostras virulentas e transmissão a animais soronegativos já foram demonstrados experimentalmente (MENGELING et al., 1999). Também se observou transmissão do vírus vacinal pelo sêmen (CHRISTOPHER-HENNINGS et al., 1997), bem como a ocorrência de infecção congênita (NIELSEN et al., 2002).

Por outro lado, um grande avanço no conhecimento da biologia do PRRSV foi alcançado com a obtenção de clones infecciosos de DNA complementar (cDNA) por meio da manipulação genômica, utilizando a tecnologia de genética reversa (MEULENBERG et al., 1998a; VAN OIRSCHOT, 2001; NIELSEN et al, 2003; TRUONG et al., 2004, KWON et al., 2006). Com o uso dessa metodologia, tem sido possível a realização de modificações pré-definidas no genoma viral (deleções, inserções e/ou substituições de nucleotídeos), possibilitando, desta forma, estudos detalhados dos mecanismos moleculares relacionados com a replicação, patogenia, persistência e imunidade (YOO et al., 2004). Além disso, a tecnologia de genética reversa permite ainda a manipulação genômica visando à produção de cepas vacinais geneticamente atenuadas ou com alterações em proteínas virais para serem utilizadas na profilaxia e controle das infecções causadas pelos arterivírus (YOO et al., 2004).

Nesse sentido, o conhecimento de genes responsáveis pela virulência bem como a identificação de proteínas e/ou epitopos imunogênicos e conservados, possuem implicações diretas na elaboração de uma nova geração de vacinas que sejam mais eficazes, seguras e que possuam um marcador sorológico, possibilitando a diferenciação entre animais vacinados e infectados naturalmente.

Vacinas diferenciais, também conhecidas como DIVA *(differentiating infected from vaccinated animals)*, consistem basicamente em vacinas produzidas pela deleção de um ou mais genes no vírus vacinal. Esse gene ausente na cepa vacinal deve codificar uma proteína

imunogênica, conservada entre isolados de campo e não essencial para a replicação viral *in vitro e in vivo*. Exemplos clássicos do uso dessa tecnologia são as vacinas amplamente utilizadas para o controle das infecções pelo herpesvírus bovino tipo 1 (BoHV-1) e pelo vírus da doenca de Aujeszki (PRV) (van OIRSCHOT et al., 1996). Quando acompanhadas de um teste sorológico que permita a detecção de anticorpos específicos contra a proteína deletada do vírus vacinal, esse tipo de vacina constitui-se em uma ferramenta de grande utilidade em programas de controle e erradicação dessas enfermidades (van OIRSCHOT, 1999).

No caso do BoHV-1 e PRV, que são vírus com um genoma DNA que codifica várias proteínas, foi possível a deleção total de genes que codificam proteínas não essenciais como a glicoproteína E (gE), sem afetar negativamente com a capacidade de replicação viral e imunogenicidade da cepa manipulada (van OIRSCHOT, 1999). No entanto, é questionável se tal procedimento seria aplicável a pequenos vírus RNA, como o PRRSV, em que aparentemente todas as proteínas possuem funções essenciais (WELCH et al., 2004; YOO et al, 2004; WISSINK et al., 2005). Assim, uma alternativa para a seleção de marcadores sorológicos a serem mutados/deletados em uma vacina atenuada para o PRRSV, seria a identificação de pequenos fragmentos (epitopos) imunogênicos em diferentes proteínas codificadas pelo vírus.

A presença de epitopos reconhecidos por linfócitos B em proteínas estruturais e não estruturais de cepas européias do PRRSV foi previamente demonstrada pelo uso de bacteriófagos expressando pequenos fragmentos de diferentes proteínas virais, (OLEKSIEWICZ et al., 2001; 2002). Entretanto, um grupo de epítopos imunogênicos identificados na proteína não estrutural Nsp2 não foi reconhecido pelo soro de animais infectados com cepas norte-americanas (OLEKSIEWICZ et al., 2001). Em um estudo subseqüente, foi demonstrada pelo mesmo grupo de pesquisadores a presença de epitopos de células B distribuídos nas ORFs 2 a 6 de um isolado europeu do PRRSV. Esses epitopos, porém, também não foram consistentemente reconhecidos pela maioria das amostras de soro testadas contra uma cepa européia e outra norte-americana do vírus (OLEKSIEWICZ et al., 2002). Esses dados, aliados a deleções espontâneas detectadas no gene da Nsp2 em isolados do tipo Europeu identificados nos EUA, sugere que essa proteína poderia representar um alvo importante para a producção de vacinas diferenciais derivadas de clones infecciosos (FANG et al., 2004).

AN et al. (2005) utilizando bacteriófagos, mapearam um epitopo de 9 aa localizado entre os aminoácidos 79 e 87 da proteína N, altamente conservado entre cepas européias e norte-americanas. Epítopos adicionais identificados através de anticorpos monoclonais contra

a proteína N também foram descritos (MEULENBERG et al., 1998b). Entretanto, não existem estudos demonstrando a presença de epítopos funcionais em proteínas codificadas por cepas Norte Americanas do PRRSV. Da mesma forma, não há dados disponíveis sobre regiões imunodominantes e conservadas que sejam reconhecidas pela resposta imune humoral de suínos infectados e que sejam dispensáveis para replicação viral *in vitro* e *in vivo*.

Assim, a identificação e posterior deleção de regiões imunogênicas da proteína não estrutural Nsp2 em um vírus vacinal, poderia resultar em um marcador ideal para o desenvolvimento de testes de diagnóstico diferencial (FANG et al., 2004). Da mesma forma, a identificação de segmentos imunogênicos e conservados presentes nas proteínas estruturais também poderiam representar alvos potenciais a serem modificados em um vírus vacinal.

CASTILLO-OLIVARES et al. (2003) demonstraram que a deleção de 46 aminoácidos do ectodomínio de uma glicoproteína do envelope (gL) de um outro arterivirus (EAV), não afetou negativamente a replicação viral e foi compatível com diferenciação sorológica entre animais vacinados e infectados com o vírus de campo através de um teste de ELISA. A possibilidade de discriminação sorológica entre animais vacinados e naturalmente infectados com amostras do genótipo I (tipo europeu) do PRRSV, foi ainda demonstrada pelo uso de um teste de ELISA contendo como antígeno apenas um peptídeo sintético correspondendo a um epítopo imunogênico e hipervariável identificado na ORF4 de uma cepa vacinal européia (OLEKSIEWICZ et al., 2005).

Outro exemplo em que a deleção de um epitopo linear imunodominante e altamente conservado não interferiu na viabilidade/replicação viral foi descrito por MEBATSION et al. (2002) pela manipulação da nucleoproteína (NP) do vírus da Doença de Newcastle (NDV). Neste estudo, além da diferenciação sorológica entre a resposta imune induzida pelo vírus mutante daquela induzida pelo vírus parental, foi possível a inserção e expressão de um epitopo imunogênico de outro vírus na mesma região genômica tolerável a deleção. Também foi demonstrada a possibilidade de desenvolvimento de uma vacina diferencial para o NDV através da construção de vírus quimeras (PEETERS et al., 2001).

Outros exemplos de vacinas diferenciais incluem vacinas de subunidade através de expressão recombinante da proteína E2 do vírus da Peste Suína Clássica (CSFV) em baculovírus (MOORMAN et al., 2000) e seu teste sorológico diferencial correspondente (FLOEGEL-NIESMANN et al., 2001). No entanto, é evidente a necessidade de uma reformulação dessas novas vacinas de subunidade, principalmente, no que se refere a sua eficácia quando comparadas às clássicas vacinas atenuadas utilizadas no controle da Peste Suína Clássica (van OIRSCHOT, 2003; van RIJN et al., 1997). Vacinas atenuadas são

geralmente aceitas como sendo mais eficazes do que vacinas inativadas em função de uma ampla estimulação do sistema imunológico e indução de imunidade duradoura. Nesse sentido, o desenvolvimento de vacinas atenuadas diferenciais contra o PRRSV representaria um avanço na produção de uma nova geração de vacinas para serem utilizadas na profilaxia e controle da enfermidade além de desejável em eventuais programas de erradicação.

Os resultados apresentados nos capítulos 2 e 3 demonstram uma alternativa para a produção de vacinas diferenciais contra o PRRSV pela identificação de marcadores sorológicos por um mapeamento de epítopos lineares de células B e posterior deleção de regiões imunogênicas em um clone infeccioso de cDNA.

3. CAPÍTULO 2

Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of Porcine Reproductive and Respiratory Syndrome virus

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Abstract

We describe B-cell linear epitopes detected by Pepscan in the Nsp2 and all of the structural proteins of a US PRRSV strain, using sera of 15 experimentally infected pigs. The Nsp2 was found to contain the highest frequency of immunodominant epitopes (n=18) when compared to structural proteins. Ten of these 18 Nsp2 peptides were reactive with 80 to 100% of the examined sera. In the structural proteins, epitopes consistently recognized by immune sera were located at Gp2 (n=2), Gp3 (n=4), Gp5 (n=3), M (n=2) and N protein (n=2). Overall, the highest degree of immunogenicity and conservation was exhibited by two epitopes identified in the C-terminal end of the M protein (ORF6). The antibodies recognizing the immunodominant epitopes of each protein were detected as early as days 7 to 15 p.i. and remained detectable until the end of the experiment (day 90 p.i). These findings have direct implications for PRRSV differential diagnostics and eventual eradication, as the identified epitopes may represent serologic marker candidates for differential (DIVA) PRRSV vaccines, derived from infectious cDNA clones.

Keywords: PRRSV; B-cell epitopes; peptides; infectious cDNA clone, pepscan.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is considered to be one of the most economically important infectious diseases of swine causing late-term reproductive failure in pregnant sows and severe pneumonia in neonatal pigs (Snijder and Meulenberg, 2001). The disease was first reported in 1989 and the causative agent was isolated and characterized for the first time in Europe in 1991 and one year later in the United States (Collins et al., 1992).

PRRSV, the etiological agent of PRRS, is an enveloped single-stranded RNA virus belonging to the order Nidovirales, family Arteriviridae (Snijder and Meulenberg, 2001). Other members of Arteriviridae are lactate dehydrogenase-elevating virus of mice (LDEV), equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV) (Snijder and Meulenberg, 2001). The PRRSV genome is approximately 15 kb in length and contains eight open reading frames (ORFs). ORFs 1a and 1b comprise 80% of the genome size and encode a polyprotein which is co- and post-translationally processed by autoproteolytic cleavage into 12 nonstructural polypeptides (Nsps). The other six ORFs (2 to 7) are translated into structural proteins (gp2, gp3, gp4, gp5, M and N proteins). An additional protein encoded by ORF2b has also been recently characterized (Lee and Yoo, 2005; Wu et al., 2001).

Significant antigenic and genetic differences have been reported among North American and European strains of PRRSV (Allende et al., 1999; Wensvoort et al., 1992). Such diversities have led to the recognition of two distinct serotypes of PRRSV: European (type 1) and North American (type 2). However, the origin of PRRSV remains unknown, especially since the European and North American PRRSV isolates cause similar clinical symptoms but represent two distinct viral genotypes with genomic divergences of approximately 40% (Nelsen et al., 1999).

Vaccination against PRRSV infections is being carried out since 1995 in the US. The most commonly used vaccine is a modified-live virus, consisting of a wt PRRSV US strain attenuated by multiple passages in cell cultures. The efficacy of these attenuated vaccines currently in use is somewhat disputed and is generally acknowledged that significant latitude still exists for technical improvements on their safety and efficacy. One technical improvement which is central to the effective implementation of these PRRSV vaccines is endowing them with differential capability to make them compatible with elimination of the wt PRRSV infection from a herd. The serological differentiation between vaccinated and naturally infected animals has proved crucial for the success of eradication programmes of important livestock diseases (van Oirschot, 1999).

Marker vaccines (also termed DIVA – Differentiating Infected from Vaccinated individuals) carry at least one antigenic protein less than the corresponding wild-type virus, i.e. has a “negative marker” (van Oirschot, 1999), which allows the serological tracing of wt strains (which obviously are “marker positive” instead) in vaccinated herds. Classical examples of modified-live vaccines carrying deletions of non-essential and immunogenic structural proteins have been produced for large DNA viruses such as pseudorabies virus (PRV) and bovine herpesvirus-1 (BHV-1) (Kaashoek et al., 1994; Moormann et al., 1990; van Oirschot, 1999). These first successful approaches to DIVA vaccines based on deletion of an entire glycoprotein from veterinary herpesvirus contributed to define two fundamental properties of an ideal serologic marker antigen: 1. the marker candidate has to be immunodominant and therefore recognized by the vast majority of a population infected with wt virus; 2. their deletion from the vaccine genetic make up would not alter the viability and/or protective immunogenicity of this vaccine. However, the applicability of such approach for small RNA viruses like PRRSV, which encode only a few proteins with essential functions, seems difficult (Welch et al., 2004; Wissink et al., 2005; Yoo et al., 2004).

Therefore, one alternative to be considered for the selection of negative serological markers for RNA viruses would be the identification of small immunogenic non essential epitopes in viral proteins. The approach of epitope deletion has proved feasible for arteriviruses through deletion of a 46 amino acid immunodominant region from the ectodomain of the glycoprotein L (gL) of EAV without deleterious effects on the replication and immunogenicity of the virus (Castillo-Olivares et al., 2003). Furthermore, a peptide ELISA based on this particular domain enabled serological discrimination between vaccinated and wild-type virus-infected animals.

The presence of B-cell epitopes in nonstructural (mainly Nsp2) and structural proteins of an EU-type PRRSV strain have been previously demonstrated by using phage display libraries (Oleksiewicz et al., 2002; Oleksiewicz et al., 2001). A few additional epitopes identified by means of PRRSV N monoclonal antibodies (Mabs) have also been reported (Meulenberg et al., 1998; Zhou et al., 2005). Nonetheless, data regarding functional epitopes on US-type PRRSV-encoded proteins are scarce. Likewise, there is no available data about immunodominant and conserved B-cell epitopes consistently recognized by the humoral immune response of US-type PRRSV infected animals. This study reports a detailed and systematic investigation by Pepscan technology of the US-type PRRSV B-cell linear epitopes recognized by the convalescent sera from PRRSV-infected pigs. Our results identify several immunodominant epitopes within the Nsp2 as well as the structural proteins of PRRSV.

Results and Discussion

Experimental inoculation

Inoculation of fifteen piglets with $10^{5.0}$ $TCID_{50}$ of a PRRS virus recovered from a NVSL 97-7895 strain full-length cDNA infectious clone by intranasal and intramuscular routes resulted in a slight increase in rectal temperature ($\leq$ 1.5ºC) between days 2 and 6 post-

infection (pi). Viremia titers ranging from $10^{3.7}$ to $10^{5.1}$ $TCID_{50}$/ml of serum was observed at 7dpi in all infected animals indicating active viral replication (data not shown). Two serum samples collected at 7dpi and all samples collected at days 15, 30, 45, 60 and 90 pi were positive for PRRSV-specific antibodies as assayed by a commercially available ELISA kit (Idexx Labs, Inc) (data not shown). The results of virus isolation from blood and ensuing positive serology demonstrated that productive virus replication and induction of a normal humoral response took place in all the experimentally infected piglets. Eight of the piglets were euthanized at 60dpi and the remaining animals (n=7) were monitored serologically until day 90 pi.

Identification of US-type PRRSV-specific linear B-cell epitopes

For the identification of US-type PRRSV-specific B-cell linear epitopes, convalescent sera (60dpi) collected from experimentally infected piglets were used for screening of the peptide-specific immune response against Nsp2 and all structural proteins. The serum samples were examined for antibodies that recognize synthetic peptides used individually as antigen in a peptide-based indirect ELISA as described in Material and Methods. The 213 synthetic peptides were designed based on the amino acid sequence of the North American strain of PRRSV NVSL 97-7895 (GenBank accession n. ***AY545985***). Peptide scanning (Pepscan) for epitope mapping has become increasingly recognized as a method for identification of diagnostically relevant epitopes within viral proteins (He et al., 2004a; He et al., 2004b; Hohlich et al., 2003; Khudyakov et al., 1999; Lundkvist et al., 1995; Niikura et al., 2003).

We identified several B-cell linear epitopes along the amino acid sequence of all the studied proteins. The identity and location of the immunodominant epitopes identified in each protein are presented in Figure 1 and in Table 1. In general, the antibodies recognizing the immunodominant epitopes appeared between days 7 and 15 pi, increased in titer with time

and remained at fairly steady levels up to day 60pi (Figure 2). Nevertheless, the antibody response to the individual epitopes varied greatly among individual pigs as measured by the optical density values of the peptide-based ELISAs (Figure 3). All seven serum samples available at 90dpi were also reactive against the immunodominant epitopes identified (data not shown).

B-cell linear epitopes are scattered along the Nsp2 amino acid sequence

The Nsp2 protein has been shown to be highly variable among arteriviruses, with similarities observed only in the amino- and carboxy terminal domains whereas the central region of the protein varies in both length and amino acid composition (Allende et al., 1999). Interestingly, the Nsp2 was found to contain the highest frequency of immunogenic epitopes when compared to the structural proteins examined in this study. Among the 97 peptides spanning the entire amino acid sequence of Nsp2, 18 were found to be immunoreactive with more than 50% of the sera tested. Ten of these peptides were reactive with 80 – 100% of the sera examined (Table 1; Figure 4A). Furthermore, the identified immunodominant B-cell epitopes were scattered along the protein sequence and most of them were localized within predicted hydrophilic regions of the protein (Figure 4B). These results were not unexpected since hydrophilic amino acid sequences are likely exposed on the surface of the protein and thus may be more easily recognized by B-lymphocytes. In addition, several other peptides were recognized by fewer serum samples. No antigenic reactivity was found within the region comprising peptides #84 and 97 located in the C-terminal end of the protein (Figure 4). The lack of reactivity of peptides spanning this region might be attributed to the high level of conservation and hydrophobicity of this segment. A previous report has also demonstrated the occurrence of a cluster of B-cell epitopes in Nsp2 of an EU-type PRRSV isolate, 111/92 (Oleksiewicz et al., 2001). However, the six epitopes identified in that study were not

recognized by antibodies from animals infected with US-type PRRSV and no comparison could then be drawn with the findings of our experiment. In addition, the systematic pepscan methodology used in our study allowed the identification of a higher number of B-cell epitopes in Nsp2 when compared to those reported for the European strain of PRRSV studies by phage display technology.

B-cell linear epitopes in the ORF2 protein

The 29-30 kDa glycoprotein 2 (gp2) and the glycoprotein 4 (gp4) are minor components of the PRRSV envelope (Snijder and Meulenberg, 2001). The antigenicity of the gp2 is largely unexplored and there is no data available regarding the North American strains. In this study, two B-cell linear epitopes were found to be immunoreactive with 60% (9/15) of the sera (Figure 5). The reactive peptides comprise regions at amino acid positions 41-55 and 121-135 within the ORF2 sequence (Table 1). Using phage-displayed peptides, Oleksiewicz, et al., (2002) identified three weakly antigenic B-cell epitopes in the ORF2 at positions 36-51, 117-139 and 120-142 of an EU-type strain. Although the epitope mapping experiments were carried out using distinct approaches and different strains, the amino acid sequence G^{123}QAAWKQVVXEAT^{135} localized in the predicted most hydrophilic domain of gp2 was identified in our study (peptide #110) as well as in that by Oleksiewicz et al., (2002). Thus, those residues might constitute the core of an epitope recognized by sera from pigs infected with EU- and US-type of PRRSV. However, it is important to consider that this region was recognized only by 1 out of 6 sera tested by Oleksiewicz et al., (2002) and by 60% of the 15 sera tested in our study, indicating a lesser immunodominance and diagnostic usefulness.

B-cell linear epitopes in the ORF3 protein

The highly glycosylated ORF3-encoded protein is the second most variable PRRSV protein, showing approximately 54 to 60% aa identity between the North American and European genotypes (Dea et al., 2000). In our investigation, four overlapping consecutive peptides (pep #129-132) were strongly immunoreactive with 85-100% of the tested sera (Table 1 and Figure 5). Those peptides cover a region comprising amino acids 61-105, which is predicted to be located in the most hydrophilic region within the ORF3 sequence. This data suggests that this region might be considered as one important immunodominant domain of the gp3 of North American strains of PRRSV. Our findings are supported by results of a recent analysis of the antigenic structure of gp3 encoded by a Chinese isolate (US-type) of PRRSV (Zhou et al., 2005). After sequential deletion of amino acid residues from each peptide, these authors found that the minimal epitopes recognized by the MAbs were localized to Y^{67}EPGRSLW^{74} and W^{74}CRIGHDRCGED^{85}. Interestingly, except for a serine instead of a glycine at position 83, identical sequences recognized by MAbs were found in the peptides #129 and #130 which were reactive with 86.7 (13/15) and 93.3% (14/15) of the swine sera examined in our study. Most importantly, a high degree of sequence conservation within a segment comprising residues 69-78 and 90-99 was observed among North American isolates and reference strains of PRRSV (Figure 6). Furthermore, in spite of the sequence variability observed among North American and European strains in this segment of gp3, Oleksiewicz et al., (2002) observed strong reactivity within a region comprising the amino acids 60-87 of an EU-type isolate of PRRSV.

B-cell linear epitopes in the ORF4 protein

The glycoprotein 4 (gp4) is a typical class I membrane protein and it is a minor constituent of the viral envelope (Meulenberg, 2000). In the present study, only a small fraction (33.3% and 26.6%) of the tested sera were found to be reactive with peptides #153

and #158 comprising amino acid residues 51-65 and 101-115 within the ORF4 protein (Figure 5). The core of a neutralization domain of the glycoprotein encoded by ORF4 of Lelystad virus and recognized by MAbs consists of amino acids 59 to 67 and is located at the most variable region of the protein (Meulenberg et al., 1997). However, further studies are necessary to demonstrate whether the linear epitope identified in our study (aa 51-65) is recognized by neutralizing antibodies. In addition, a single linear epitope in gp4 (aa 59-71) encoded by a European strain was found to be immunodominant in pigs and a putative decoy function for this region has been suggested (Oleksiewicz et al., 2001; Oleksiewicz et al., 2005). However, we did not detect immunodominant sequences in this region (peptide #153), since it was only recognized by only 5 out of 15 tested sera.

B-cell linear epitopes in the ORF5 protein

Glycoprotein 5 (gp5) is one of the major structural proteins encoded by PRRSV and forms disulfide-linked heterodimers with M protein in the viral envelope (Snijder and Meulenberg, 2001). Specific IgG antibodies to gp5 are detected at the end of the first week after infection, and at around 14 dpi to M protein (Dea et al., 2000). A neutralizing epitope (epitope B) in the ectodomain of gp5 has been previously described (Ostrowski et al., 2002). The core sequence of this neutralizing epitope “B” (H^{38}, Q^{40}, I^{42}, Y^{43} and N^{44}) is present in our peptide #168 that was found to be reactive with 8 out of 15 sera. However, since neutralizing activity was observed in all of the 15 sera samples used in our experiments (including the seven sera which did not react with peptide #168, data not shown), one might speculate that, besides epitope B, there maybe other neutralizing epitopes present on PRRSV, that would contribute to the total PRRSV-neutralizing activity in the serum. In addition, the peptide comprising the residues 187-200 (pep#184) located in the 3’ endodomain of the protein was recognized by 13/15 of the examined sera. The aa sequence

P^{188}LTR(V/T)SAEQW^{197} was also found to be reactive with sera raised against an European PRRSV strain (Oleksiewicz et al., 2002). Surprisingly, peptide #184 exhibited the lowest value of the mean optical densities obtained among all the immunodominant epitopes identified (Figure 3). Despite some amino acid changes within this region and the low immunogenicity observed, this decapeptide is relatively well conserved among North American strains (Figure 6). Most importantly, this peptide was recognized by the majority of the animals used in our experiment. This inverse correlation between immunogenicity and level of sequence conservation has been previously described (Oleksiewicz et al., 2001).

B-cell epitopes in the ORF6 protein

The nonglycosylated M protein (16-20 kDa) is the most conserved structural protein of arteriviruses. Abundant molecules of M protein are present in the virion associated with gp5 and its N-terminal half presumably traverses the envelope membrane three times (Dea et al., 2000; Snijder and Meulenberg, 2001). The protein has only a short stretch of 10 to 18 residues exposed at the virion surface and a large endodomain (Dea et al., 2000; Snijder and Meulenberg, 2001). In this study, we demonstrated that peptides #200-201 constitute a unique combination of sequence conservation and antigenicity (Figures 5 and 6). These peptides were found to be reactive with 100% of sera examined. These immunoreactive peptides are located within the region at position 151-174 which corresponds to the C-terminus end of the endodomain of M protein. Eventually, the 5aa (A^{161}VKQG^{165}) could be considered the minimal sequence recognized by antibodies once this segment correspond to the overlapping residues of the peptides #200 and #201. (Oleksiewicz et al., 2002) also reported the identification of one phage-displayed epitope localized in the large putative endodomain of the M protein (aa 138-159) of a European-type strain. In their experiment, this epitope was recognized by a reduced number of sera collected very late in infection. In the present study, the overlapping peptides were reactive with 100% of the sera examined. The identified

epitopes were found to be highly immunogenic and conserved among isolates and reference strains of both PRRSV genotypes (Figure 6). In order to confirm the results demonstrated by the sequence alignment, we further tested the reactivity of both synthetic peptides with sera from 21 pigs experimentally infected with the homologous (NVSL 97-7895) PRRSV strain and antisera raised against four heterologous US strains. As expected, all sera samples were confirmed positive (data not shown). Reactivity of these two synthetic peptides with a sizable number of field sera was also observed (data not shown). In addition, antiserum against Lelystad virus (European prototype strain of PRRSV) was also reactive with peptide #201. These results demonstrate that the peptide containing the residues $A^{161}VKQGVVNLVKYAK^{174}$ can be particularly useful for diagnostic purposes and one attractive candidate to be evaluated as a negative serological marker in a PRRSV vaccine derived from infectious cDNA clones, if proved to be dispensable from the ORF6 without affecting the viability of the vaccine strain and/or protective immunity induced by it.

B-cell epitopes in the ORF7 protein

After infection, most PRRSV-specific IgG antibodies are primarily directed against the nucleocapsid (N) protein and are detectable as early as 7dpi. Thus, these antibodies may be useful for diagnostic purposes (Dea et al., 2000). A commonly used commercial PRRSV ELISA kit contains N protein as the single antigen for serological diagnosis of PRRSV infections. Our epitope mapping has demonstrated that among the 12 peptides derived from the N protein amino acid sequence, six were found to be immunoreactive. Two out of these six peptides (pep #203, aa 11-25 and #206, aa 41-55) located at predicted hydrophilic domains of the protein were reactive with 14/15 and 7/15 of the sera, respectively (Figure 5). Furthermore, we observed that residues G^{18}(N/D)$GQPVNQ^{25}$ contained in the peptide #203, which was recognized by 93.3% of the animals, were well conserved among 33 isolates and

reference strains from both PRRSV genotypes (Figure 6). Recently, An et al. (2005) using a phage-display peptide library, identified a well conserved B-cell epitope with an anti-N protein MAb. The core sequence recognized by this MAb comprised the residues $I^{79}QTAFNQGA^{87}$ in the context of the N protein. No reactivity was observed within that region probably because no peptide examined in our study contained the minimal sequence previously identified as a B-cell epitope.

Antibodies recognizing the immunodominant epitopes of Nsp2 and structural proteins appear between days 7 and 15 pi and remain detectable until at least 90 dpi

Serum samples collected at different time points after experimental infection were used to study the seroconversion kinetics to the immunodominant epitopes identified in Nsp2 and structural proteins of the NVSL 97-7895 strain. Animals seroconverted to the immunogenic B-cell epitopes at different times post-infection ranging from 7 to 45 dpi. Seroconversion kinetics revealed that peptide-specific antibodies started appearing generally between days 7 and 15 post-infection, increased in titer with time and remained at fairly steady and high levels up to day 60pi (Figure 2). In addition, reactivity was also detected against all the identified epitopes with sera collected at 90dpi, although a slight decrease in the OD values could be observed when compared to those recorded at 60 dpi (not shown). Furthermore, the antibody response to some Nsp2 epitopes seemed to appear slightly earlier in some animals when compared to the response against the structural proteins. However, the seroconversion kinetics to the immunodominant epitopes was not able to discriminate between the serological response to Nsp2 and structural proteins.

Significance of the epitope information

The feasibility of a new strategy for designing marker vaccines based on the deletion of immunodominant epitopes has been recently demonstrated for RNA viruses (Castillo-Olivares et al., 2003; Mebatsion et al., 2002). However, the possibility of using such approach for the development of PRRSV vaccines remains to be explored yet. Likewise, information regarding immunogenicity and presence of B-cell epitopes, which could be used as serological marker candidates, in different US-type PRRSV proteins are scarce. Fang et al. (2004) identified natural deletions within Nsp2 gene of European-like PRRSV isolated in the United States, suggesting that this protein could represent an ideal target for the development of marker vaccines. The data presented in our paper indicate the presence of several B-cell epitopes distributed along the amino acid sequences of Nsp2 and structural proteins of the North American strain NVSL 97-7895, which served as basis for the construction of an infectious full-length cDNA clone of PRRSV (Truong et al., 2004). Additionally, several epitopes (especially those found in ORF6) were found to be highly immunogenic, consistently recognized by the 15 PRRSV-infected pigs and well conserved among North American and European strains of PRRSV. To our knowledge, this is the first report demonstrating the presence of B-cell linear epitopes consistently recognized by immune serum from pigs experimentally infected with US-type PRRSV.

The detailed and systematic methodology employed in this study by using overlapping synthetic peptides, enabled us to identify a higher frequency of B-cell linear epitopes (mainly in Nsp2) in comparison to previous findings reported for EU-type PRRSV strains. The identification of conserved and antigenic peptides corresponding to B-cell epitopes consistently recognized by PRRSV-infected animals may have major practical significance by providing the molecular basis for development of improved diagnostic tests as the identified

epitopes may be considered serological marker candidates for differential (infection vs. vaccination) marker PRRSV vaccines derived from infectious cDNA clones.

Finally, it must be born in mind that some of the immunodominant B-cell epitopes we identified on the PRRSV proteins may have a role in PRRSV-neutralizing activity and therefore on PRRSV protective immunity. It will be interesting to investigate if additional epitopes other than “B” epitope on GP5 and perhaps in other glycoproteins and M protein have a role in neutralization. Such quality, although important for protection and vaccine design, could recommend against their use as deletable serologic differential markers.

Material and Methods

Cells and virus

Infectious PRRSV virus (US-type) recovered from MARC-145 cells transfected with *in vitro* produced transcripts of the full-length cDNA clone (FL12) of PRRSV NVSL 97-7895 (Truong et al., 2004) was used for animal inoculation and antiserum production. The cells were propagated in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin, 20µg/ml of streptomycin and, 20µg/ml of kanamycin). These cells were used for electroporation of RNA, viral infection and growth and for virus titration.

Animal inoculation

Fifteen mixed-breed (Landrace x Large White) piglets averaging 3 weeks of age were obtained from a PRRSV-free farm. The animals were allocated in three BL-2 isolation rooms and inoculated with a total dose of $10^{5.0}$ $TCID_{50}$/3ml of PRRSV FL12 by intranasal (1ml in each nostril) and intramuscular (1ml) routes. The animals were clinically monitored on a daily

basis and their rectal temperatures were recorded from 2 days pre-inoculation to day 15 post-infection (pi). Sequential blood samples were collected from all animals at days 0 (zero), 7, 15, 30, 45, 60 and 90 pi and tested for PRRSV-specific antibodies by using a commercially available ELISA kit (Idexx Labs, Inc).

Synthetic peptides

A set of 213 overlapping 15-mer synthetic peptides, which overlapped each other by 5 aa, spanning the entire amino acid sequence of the non-structural protein Nsp2 (n=97) and all structural proteins (ORF2, n=25; ORF3, n=25; ORF4, n=17; ORF5, n=20; ORF6, n=17 and ORF7, n=12) of the North American strain of PRRSV (NVSL 97-7895) were used individually in a peptide-based enzyme-linked immunosorbent assay. Peptides were synthesized using Fmoc solid-phase chemistry by Open Biosystems, Inc, Huntsville AL.

Peptide ELISA

Serum samples collected at day 60 pi from the 15 piglets experimentally infected with FL-12 strain were used for screening of the peptide-specific antibody response by ELISA. Briefly, Immulon 2HB flat bottom microtiter 96 well plates (Thermo Electron, Milford, MA) were coated with 100µl of a peptide solution (10µg/ml) in 0.1M carbonate buffer (pH 9.6), and incubating overnight at 4ºC. After blocking with 250µl of a 10wt. % nonfat dry milk solution for 4h at room temperature on a plate shaker, the plates were washed three times with PBS containing 0.1% Tween 20 (PBST-20). Unbound reagents were further removed by striking the plates repeatedly, bottom up, on a stack of absorbent paper towel. Then, 100µl of pig sera (1:20) diluted in 5wt. % nonfat dry milk in PBST-20 was added per well and plates were incubated in the shaker for 1h at room temperature. After washing five times with PBST-20, each well received and was incubated with 100µl of the affinity purified antibody

peroxidase labeled goat anti-swine IgG (KPL, Gaithersburg, MD) diluted 1:2000 in PBST-20 with 5wt. % nonfat dry milk for 30min at room temperature. Following a final wash, 100µl of ABTS (KPL) peroxidase substrate was added for 15 min at 37ºC and the reaction was stopped by adding 100µl of SDS 1%. A 12-mer synthetic peptide (YKNTHLDLIYNA) which has been shown to be recognized by PRRSV neutralizing antibodies (Ostrowski et al., 2002) served as a positive peptide control. Serum samples collected at day 0 (zero) were used as negative control. Serum was considered positive when the OD value was above the cutoff point (the mean OD absorbance at 405nm of the negative sera plus 3 standard deviations). The same experimental conditions were applied to ELISAs conducted with each one of the 213 synthetic peptides used in this study.

Seroconversion kinetics

The seroconversion kinetics to the immunonodominat epitopes identified in Nsp2, Gp3 (ORF3), Gp5 (ORF5), M protein (ORF6) and N protein (ORF7) was examined by using sequential serum samples collected from the infected piglets at days 0, 7, 15, 30, 45 and 60dpi on the peptide-based ELISA. The serum was considered positive when the OD value was above the cutoff point.

Bioinformatics analysis

Hydropathic profiles were produced by the ProtScale program (http://us.expasy.org) using parameters defined previously (Hoop and Woods, 1981). Window sizes of 9 and 21 were used for all the structural proteins and for Nsp2, respectively. Multiple alignment of amino acid sequences were made using ClustalW (Thompson et al., 1994). Alignments were retrieved and analyzed by Bio-Edit sequence alignment editor v. 7.0.5.

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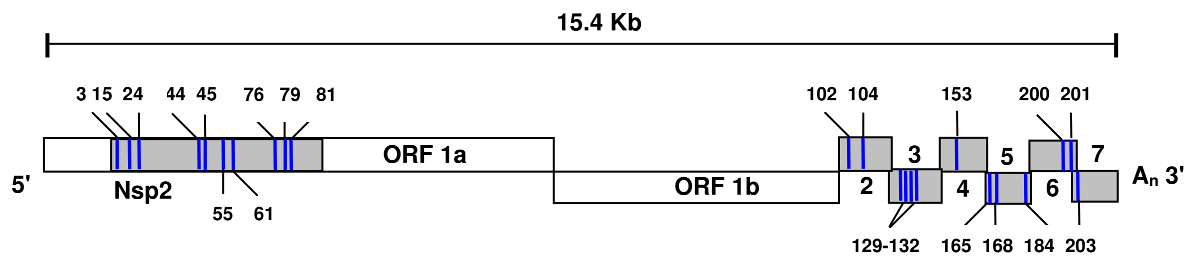

Figure 1. Position of the immunodominant B-cell linear epitopes identified on the Nsp2 and structural proteins of PRRSV. The locations with the respective ORFs and identity number of the major synthetic peptides identified as B-cell epitopes in each protein are indicated. See Table 1 for the amino acid position of each identified epitope within the sequence of the respective ORF.

Table 1. Immunodominant B-cell linear epitopes identified on Nsp2 and structural proteins of a North American strain of PRRSV. The sequence of the immunoreactive synthetic peptides and the number of seropositive animals are indicated.

Peptide n°/ protein	Amino acid sequence	Position aa[1]	N° of reactive sera[2]
3 - Nsp2	ALPAREIQQAKKHED	21-35	10/15
4 – Nsp2	KKHEDAGADKAVHLR	31-45	8/15
15 – Nsp2	ECVQGCCEHKSGLGP	141-155	14/15
24 – Nsp2	LCQVVEECCCHQNKT	231-245	14/15
44 – Nsp2	PPPPPRVQPRKTKSV	431-445	14/15
45 – Nsp2	KTKSVKSLPGNKPVP	441-455	15/15
48 – Nsp2	PDGREDLTVGGPLDL	476-490	8/15
50 – Nsp2	PMTPLSEPALMPALQ	496-510	10/15
54 – Nsp2	VTPLSEPIFVSAPRH	536-550	10/15
55 – Nsp2	SAPRHKFQQVEEANL	546-560	14/15
58 – Nsp2	ASSQTEYEASPLTPL	576-590	9/15
59 – Nsp2	PLTPLQNMGILEVGG	586-600	10/15
61 – Nsp2	VLSEISDTLNDINPA	606-620	13/15
76 – Nsp2	VPRILGKIENAGEMP	756-770	12/15
79 – Nsp2	QPVKDSWMSSRGFDE	786-800	15/15
81 – Nsp2	SAGTGGADLPTDLPP	806-820	15/15
82 – Nsp2	TDLPPSDGLDADEWG	816-830	10/15
83 – Nsp2	ADEWGPLRTVRKKAE	826-840	9/15
102 – ORF2	LPSLAGWWSSASDWF	41-55	9/15
110 – ORF2	KAGQAAWKQVVSEAT	121-135	9/15
129 – ORF3	QAAAEVYEPGRSLWC	61-75	13/15
130 – ORF3	RSLWCRIGHDRCSED	71-85	14/15
131 – ORF3	RCSEDDHDDLGFMVP	81-95	15/15
132 – ORF3	GFMVPPGLSSEGHLT	91-105	15/15
153 – ORF4	SCLRHGDSSSQTIRK	51-65	5/15
165 – ORF5	MLGRCLTAGCCSRLL	1-15	7/15
168 – ORF5	ANSNSSSHLQLIYNL	31-45	8/15
184 – ORF5	TPLTRVSAEQWGRL	187-200	13/15
200 – ORF6	LKSLVLGGRKAVKQG	151-165	15/15
201 – ORF6	AVKQGVVNLVKYAK	161-174	15/15
203 – ORF7	MPNNNGKQQKKKRGN	11-25	14/15
206 – ORF7	PGKKIKNKNPEKPHF	41-55	7/15

[1] Localization of the peptide within the amino acid sequence of the respective ORF
[2] Number of reactive sera in the peptide-ELISA. The reactivity of 15 sera was examined against each peptide.

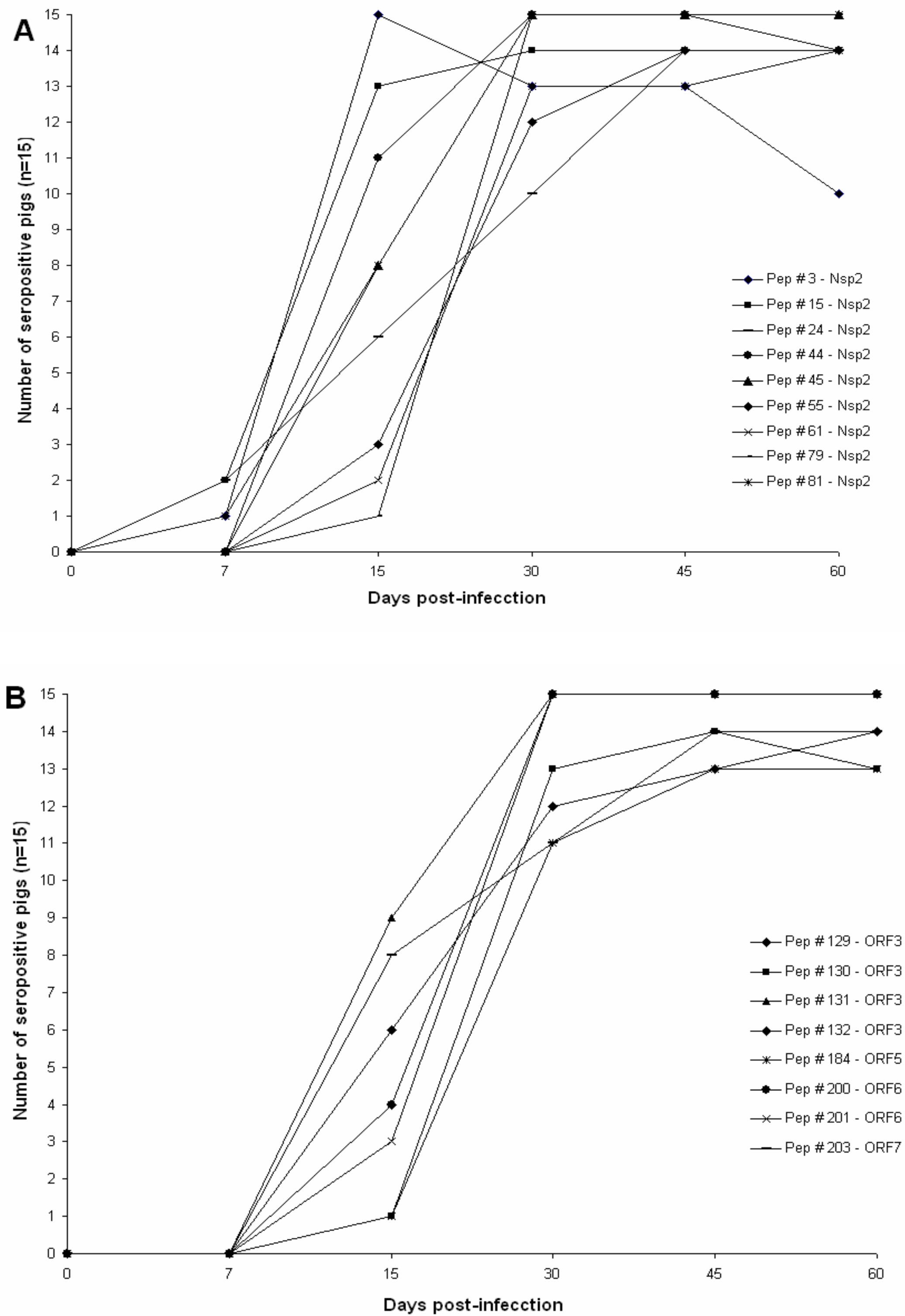

Figure 2. Seroconversion kinetics of 15 experimentally infected pigs against PRRSV specific B-cell epitopes identified on Nsp2 (A) and structural proteins (B) of a North American strain. Serum samples collected at 0, 7, 15, 30, 45 and 60 dpi were examined by peptide ELISA against all the immunodominant epitopes identified.

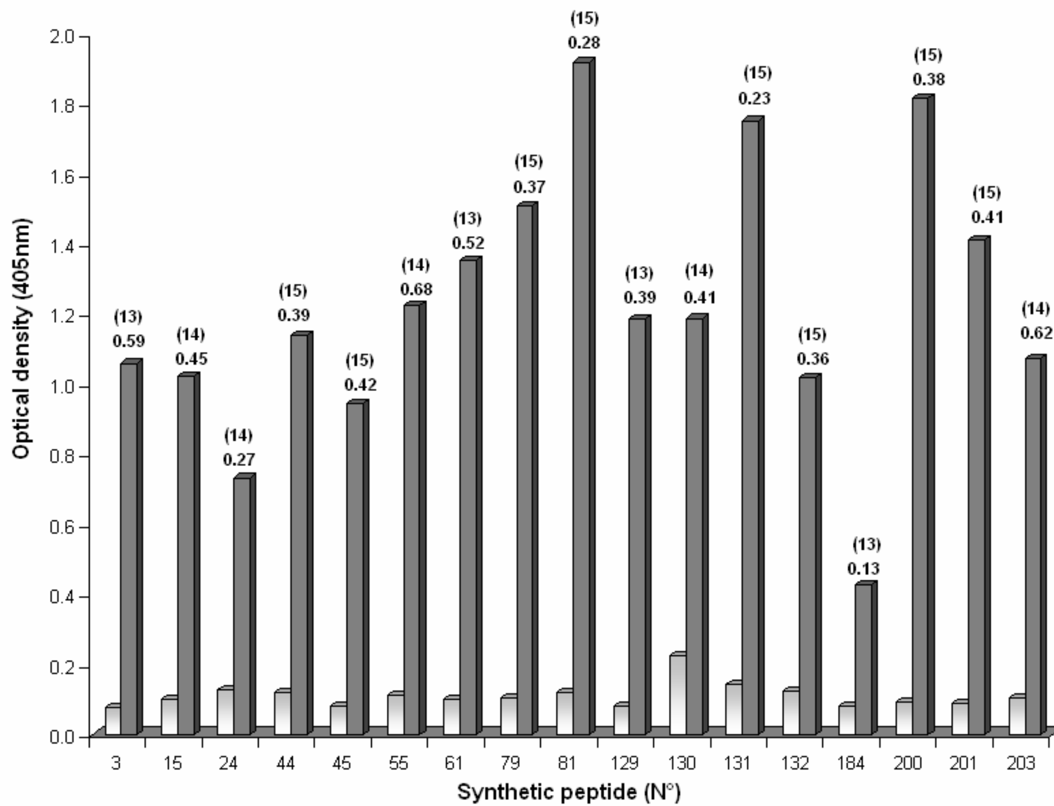

Figure 3. Mean optical density (OD) scored by ELISA test using synthetic peptides recognized by the majority of the sera at 0 (open bars) and 60dpi (darkened bars). The two decimal values located above the bars indicate the standard deviations of the seropositive samples while the number in parentheses correspond to the number of reactive sera with each peptide (total of 15 sera examined). Peptides #3 to 81 (Nsp2); #102 and 110 (ORF2); #129 to 132 (ORF3); #184 (ORF5); #200 and 201 (ORF6) and # 203 (ORF7). Refer to table 1 for exact location of the peptides.

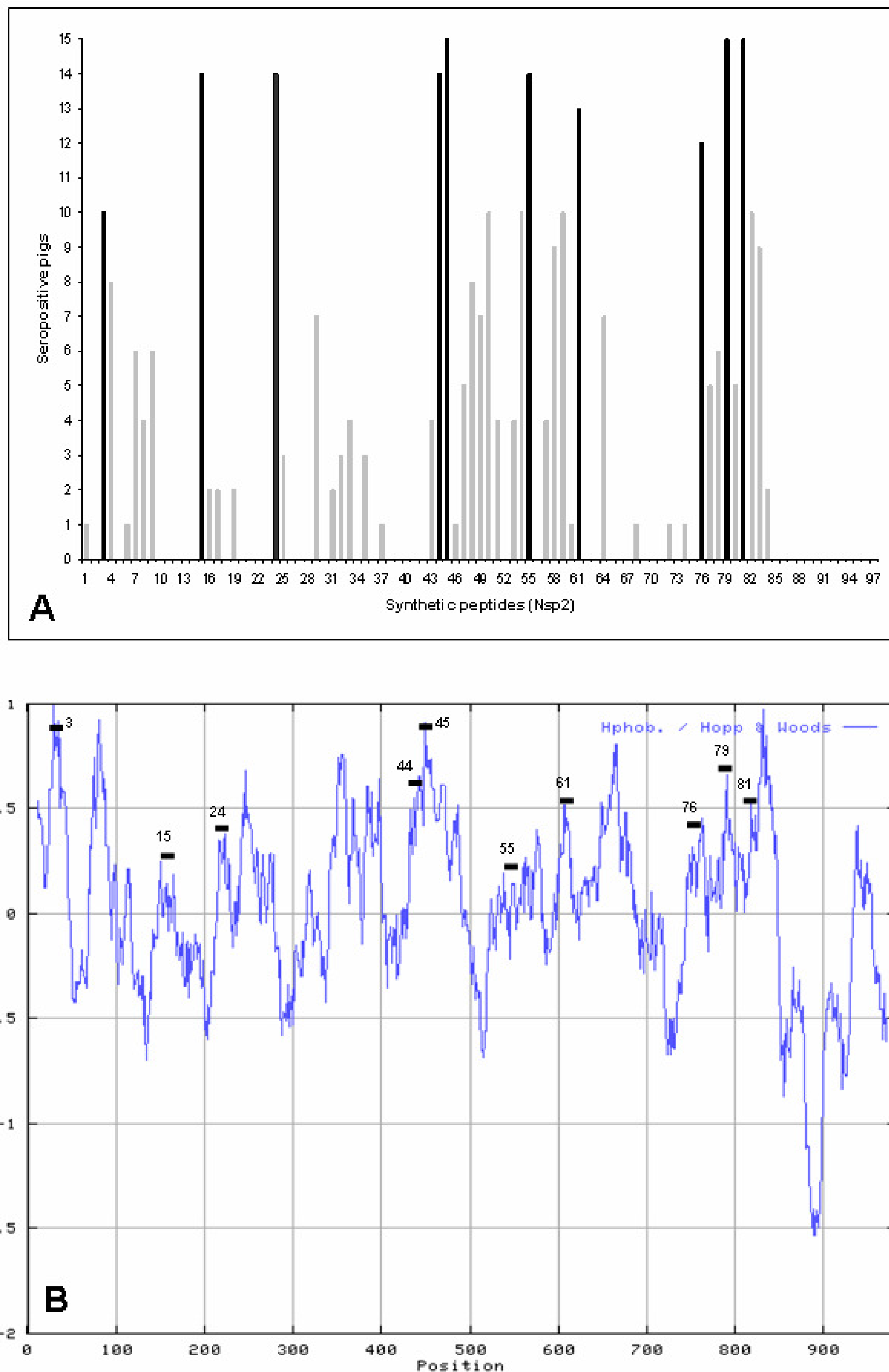

Figure 4. (A). B-cell linear epitopes on the Nsp2 amino acid sequence of a North American strain of PRRSV identified by Pepscan analysis. Black bars represent the immunodominant epitopes identified. Sera were considered positive when the OD values were above the cutoff point (the mean OD of absorbance at 405nm of the negative sera plus 3 standard deviations). (B). Hydropathic profile of the protein which was generated by the ProtScale program (http://us.expasy.org) with window size of 21 and using parameters defined previously (Hoop and Woods, 1981).

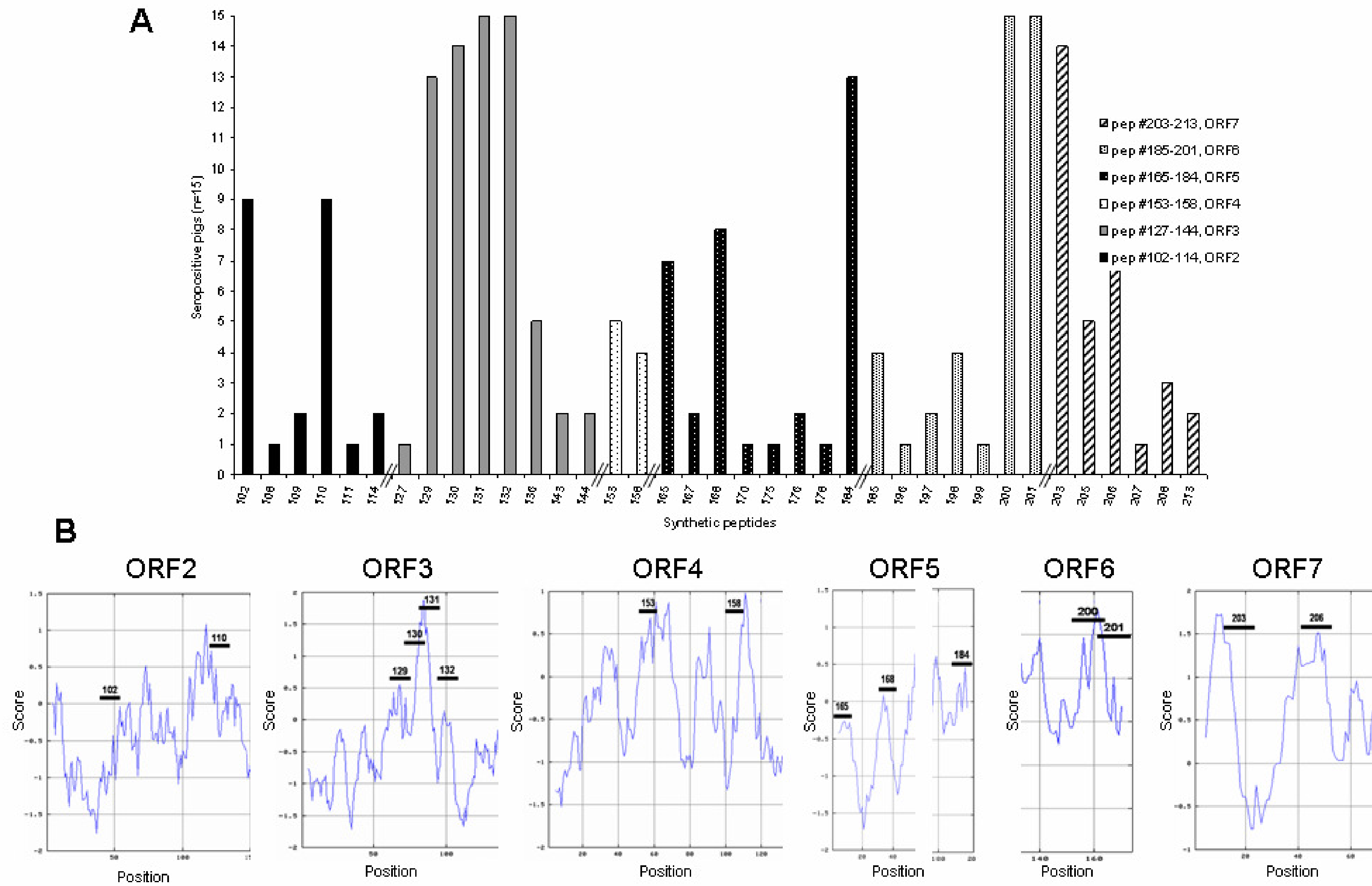
A
Seropositive pigs (n=15)
Synthetic peptides
pep #203-213, ORF7
pep #185-201, ORF6
pep #165-184, ORF5
pep #153-158, ORF4
pep #127-144, ORF3
pep #102-114, ORF2
B
ORF2
ORF3
ORF4
ORF5
ORF6
ORF7
Score
Position

Figure 5. (A) B-cell linear epitopes identified along the structural proteins (ORFs 2-7) of a North American strain of PRRSV by Pepscan analysis. The numbers of the corresponding peptides in each ORF are indicated. Sera were considered positive when the OD values were above the cutoff point (the mean OD of absorbance at 405nm of the negative sera plus 3 standard deviations). (B) Hydropathic profiles of the proteins which were generated by the ProtScale program (http://us.expasy.org) with window size of 9 and using parameters defined previously (Hoop and Woods, 1981). The localization of the immunodominant epitopes is indicated.

ORF3 (Pep # 129-132)

```
                 60        70        80        90        100
                 ....|....|....|....|....|....|....|....|....|
NVSL97-7895      QAAAEVYEPGRSLWCRIGHDRCSEDDHDDLGFMVPPGLSSEGHLT
JA-142           .............................................
HB-1_sh/2002     ......................G.....E................
25544            .....I..................G...E..........N.....
NVSL-14          .....A............Y...G.....E................
GDZC1            .....I.V..............G.N...E................
P129             ......L...............G.....E............S...
16244B           ...T.I................G.....E................
NC_001961        ...T.I................G.....E................
PL97-1           ...T.I............Y...E.....E................
PL97-1/LP1       ...T.I............Y...E.....E................
BJ-4             ...T.I............Y...E.....E................
PA8              ...T.I............Y...E.....E................
RespPRRS-MLV     ...T.I............Y...E.....E....I...........
VR-2332          ...T.I............Y...G.....E....I...........
HN-1             .....I....G.......Y...E...R.E................
NADC-9_E_        ......................G.....E...V............
FJ-1             .....I................E.....E......S......R..
LV4.2.1          ...RQRL....NM..K......E.R...E.LMSI.S.YDN-LK.E
NY4              ...YQRL....NM..K......E.R...E.LMSI.S.YDN-LK.E
EuroPRRSV        ...LQRL....NM..K......E.R.Q.E.LMSI.S.YDN-LK.E
SDPRRS04-48      ...RQRL....NM..K......E.R...E.LMSI.S.YDN-LK.E
MN-04-09_EU      ...HQRL....NM..K......E.R...E.LMSI.S.YDN-LK.E
SD-01-07         ...HQRL....NM..K......E.R...E.VMSI.S.YDN-LK.E
Consensus        *** :   ** .:**:**:*** * *:*:* : :*.* .. . :*
```

In the NVSL97-7895 row the residues QAAAEVYEPGRSLWCRIGHDRCSEDDHDDLGFMVPPGLSSEGHLT are printed in bold and underlined.

ORF5 (Pep # 184)

```
                 180       190       200
                 |....|....|....|....|
BJ-4             VLDGSVATPITRVSAEQWGRP
RespPRRS-MLV     .....................
PL97-1           .....................
PL97-1/LP1       .....................
PA8              .....................
16244B           .....................
HN1              .....................
FJ04A            .....................
NADC-8_E_        .....A...V...........
1530B            ...........K.........
NVSL97-7895      .........L..........L
JA142            .........L..........L
JA142            .........L..........L
17198-6          .........L...........
NVSL-14_E_       .....A...L...........
P129             .........L..........L
SDSU73           .....A...L..........L
HB-1_sh_/2002    .........L..........L
SDPRRS04-48      ..E.VK.Q.L..T.....EA-
MN-04-09_EU      ..E.VK.Q.L..T.....EA-
MN-03-10_EU      ..E.VK.Q.L..T.....EA-
EuroPRRSV        ..E.VK.Q.L..T.....EA-
LV4.2.1          ..E.VK.Q.L..T.....EA-
111/92           ..E.VK.Q.L..T.....EA-
Consensus        *:***:*  * *:*: *:*:*
```

In the BJ-4 row the residues TPITRVSAEQWGRP are printed in bold and underlined.

ORF6 (Pep #200-201)

```
                 140       150       160       170
                 |....|....|....|....|....|....|....
HB-2_sh/2002     STTVNGILVPGLKSLVLGGRKAVKQGVVNLVKYAK
CH1a             ......T......G.....................
P129             ......T............................
NVSL97-7895      ......T............................
JA142            ......T............................
JA-142           ......T............................
ISU-79           ......T............................
1530 B           ......T............................
NADC-9_E_        ......T............................
NC_001961        ......T............................
16244B           ......T............................
FJ-1             ......T............K...............
IAF94-287        ......T................R...........
RespPRRS-MLV     ......T............................
01NP1.2          ......T............................
BJ-4             ......T............................
HN1              ......T............................
PL97-1           ......T............................
PL97-1/LP1       ......T............................
VR2332           ......T............................
PA8              ......T............................
25544            ......T............................
8981             ......T............................
AF184212-SP      ......T.................R..........
GDCZ1            ......T............................
NVSL-14          ......T............................
ONT-TS           ......T...........................R
ISU-3927         ......T.....R......K...............
Lelystad         L.S...T.....R......KR...R........GR
LV4.2.1          L.S...T.....R......KR...R........GR
EuroPRRSV        L.S...T.....R......KR...R........GR
SD-02-10         L.S...T.....R......KR...R........GR
111/92           L.S...T.....R......KR...R........GR
Consensus        .*:*** *****:.*****::**::********.:
```

(ORF7) Peptide # 203

```
                 10        20        30        40
                 |....|....|....|....|....|....|....|
NVSL97-7895      NGKQQKKKR----GNGQPVNQLCQMLGKIIAQQNQS
JA142            .........----.......................
SDSU73           ........K----.......................
16244B           ......R.K----.D.....................
RespPRRS         ......R.K----.D.....................
PA8              ......R.K----.D.....................
BJ-4             ......R.K----.D.....................
VR2332           ......R.K----.D.....................
PL97-1/LP1       ......R.K----.D.....................
19aPP1           ......R.K----.D.....................
01NP1.2          ......R.K----.D.....................
NC_001961        ......R.K----.D.....................
17198-6          ....L.R.K----.D..........P.........P
HN1              ......R.K----.D.....................
HB-2sh/2002      ......R.K----.D.....................
ISU-P            ......R.K----.D..................H..
93-27687         ......R.K----.D.....................
92-6725          ......R.K----.D.....................
IA-D21           ........K----.......................
29D1             ........KQ---.......................
28523            ........K----.......................
93-6351          ........K----.......................
91-46907         ..R.....K----.D.....................
92-01205         ..R.....K----.D.....................
93-47324         ..R..R..K----.D.....................
MN-184           ..R.....K----.D............R........
27E              ........K----.D..................S..
P129             ........K----.......................
Lelystad         .QS.K...STAPM...........L..AM.KS.R.-
LV4.2.1          .QS.K...STAPM...........L..AM.KS.R.-
SDPRRS02-11      .QS.K...STAPM...........L..AM.KS.R.-
EuroPRRSV        .QS.K...STAPM...........L..AM.KS.R.-
111/92           .QS.K.R.NTAPM...........L..AM.KS.R.-
Consensus        :*  * ::*     *:**.******: * :* .* *.
```

Figure 6. Multiple alignment of ORFs 3, 5, 6 and 7 among North American- and European-type PRRSV isolates. The amino acid sequences of the immunodominant epitopes identified in each ORF are underlined. Alignments of the amino acid sequences were made using ClustalW (Thompson, 1994) and then the results were retrieved and analyzed by Bio-Edit sequence alignment editor v. 7.0.5.

4. CAPÍTULO 3

Use of reverse genetics to develop a live attenuated PRRSV differential vaccine: proof of concept and pursuit of an optimal marker

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Abstract

The availability of a DIVA (differentiating infected from vaccinated individuals) vaccine is crucial for the control and eradication of porcine reproductive and respiratory syndrome (PRRS). Epidemiological and regulatory considerations dictate that a PRRSV DIVA vaccine should be designed based on a negative marker (i.e., a marker absent from the vaccine strain but consistently present in wild-type isolates). While technically straightforward for some dsDNA viruses, deleting antigen-coding sequences from the genome of a live-attenuated RNA virus vaccine is rather more difficult. Previous studies in our laboratory identified several B-cell linear epitopes consistently recognized by convalescent sera obtained from pigs infected with a North American PRRSV strain. Based on their immunodominance and level of amino acid (aa) conservation, we selected two target epitopes (serological marker candidates) in each of the following PRRSV proteins: Nsp2, GP3 and M to be deleted in the wild-type cDNA infectious clone (FL-12) by site-directed mutagenesis. In the case of NSP2 protein (predictably, the viral protein most likely to tolerate large deletions) we were able to successfully rescue a mutant that fulfilled the requirements for a DIVA marker vaccine virus such as efficient growth *in vitro* and *in vivo* and induction of active seroconversion as measured by a commercial ELISA kit, with absence of a marker-specific peptide-ELISA response in 100% (n=15) of the vaccinated animals. Deletions of previously identified peptide marker candidates within GP3 and M genes were shown to be lethal for virus viability *in vitro*. Alternatively, by substitution of 5aa at a time within a M peptide marker candidate (most conserved among our candidates), we could recover a viable mutant virus, although it still resulted in a “positive marker” virus. In summary, our results provide proof of concept that DIVA PRRSV vaccines can be developed using such approach. Efforts will now focus on identifying an optimal marker or a combination of peptide markers capable

of reliably inducing a consistent serologic response in the diverse universe of wild type PRRSV strains.

Keywords: PRRSV; B-cell epitopes; peptides; infectious cDNA clone; site-directed mutagenesis; DIVA; marker vaccines.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small enveloped, positive-strand RNA virus associated with reproductive failure in pregnant sows and severe pneumonia in neonatal pigs [1]. Porcine reproductive and respiratory syndrome (PRRS) is currently one of the most important infectious diseases of swine causing significant economic losses to the pig industry worldwide [2].

PRRSV, along with lactate dehydrogenase-elevating virus of mice (LDEV), equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV), is classified into the order *Nidovirales*, family *Arteriviridae* [1]. Based on significant antigenic and genetic differences reported among North American and European PRRSV strains [3,4], two distinct genotypes of the virus have been recognized: European (type 1) and North American (type II). Although isolates from both genotypes can cause disease with similar clinical signs, their genomes exhibit divergences of approximately 40% [5].

Vaccination against PRRSV infections is being carried out since 1995 in the US. The most commonly used vaccine contains a North American PRRSV strain attenuated by multiple passages in cell cultures. The efficacy of these currently used attenuated vaccines is somewhat controversial and is generally accepted that there is a need for improvements on their safety and efficacy. In this context, the availability of a DIVA (Differentiating Infected from Vaccinated Animals) vaccine would be of great value for the control and eventual

eradication of PRRS. Epidemiological as well as regulatory considerations dictate that a PRRSV DIVA vaccine should be designed based on a negative marker (i.e., a marker absent from the vaccine strain but consistently present in wild-type strains). Classical examples of modified-live vaccines carrying deletions of non-essential and immunogenic structural proteins have been produced for large DNA viruses such as pseudorabies virus (PRV) and bovine herpesvirus-1 (BHV-1) [6-8]. While technically straightforward in the case of some double-stranded DNA viruses, deleting antigen-coding sequences from the genome of a small RNA virus like PRRSV, which encode only a few proteins with essential functions, seems a more difficult task [9-11]. Thus, the generation of a mutant virus carrying a deletion of an immunodominant and conserved protein segment (or a combination of deletions within the same protein or even in different proteins) would be an attractive alternative to generate a live-attenuated marker vaccine strain.

The presence of numerous B-cell linear epitopes consistently recognized by convalescent serum of pigs infected with PRRSV has been previously described in our laboratory by Pepscan analysis of the Nsp2 and structural proteins encoded by a North American strain of PRRSV [12]. Based on the immunodominance and level of amino acid conservation observed for some of the peptides distributed in the different proteins, we selected several target epitopes (serological markers candidates) to be deleted in the wild-type infectious cDNA clone (FL-12) by site-directed mutagenesis.

The approach of epitope deletion has proved feasible for arteriviruses through deletion of a 46 amino acid immunodominant region from the ectodomain of the glycoprotein L (gL) of EAV without deleterious effects on the replication and immunogenicity of the virus [13]. Furthermore, a peptide ELISA based on this particular domain enabled serological discrimination between vaccinated and wild-type virus-infected animals [13].

The present study describes the generation of a PRRSV deletion mutant, named FL-dNsp2/44, which lacks amino acid residues 431 to 445 within the sequence of the Nsp2 derived from a US-type strain of PRRSV. In order to explore the potential of this approach to generate a live-attenuated marker vaccine against PRRSV, we evaluated the replication efficacy *in vitro* of the epitope deletion mutant and its biological properties *in vivo,* such as replication efficiency and immunogenicity in pigs as well as virulence in a pregnant sow model. On the other hand, attempts to delete selected epitopes in Gp3 and M protein were unsuccessful, since the removal of these regions was lethal for virus viability. In addition, amino acid substitutions within the most conserved epitope in the M protein resulted in a viable mutant, despite its marker positive feature.

2. Material and Methods

2.1. Cells, viruses and antibodies

The virus used for animal inoculation was recovered from MARC-145 cells transfected with RNA transcripts produced *in vitro* from the full-length infectious cDNA clone (FL12) derived from PRRSV NVSL 97-7895 type II strain (GenBank accession n. ***AY545985***) [14]. MARC145 cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin, 20µg/ml of streptomycin and, 20µg/ml of kanamycin) and used for RNA electroporation, viral infection and amplification, virus titration and experiments of growth kinetics. Immunofluorescence indirect assays (IFA) were performed as previously described [15], using N protein-specific monoclonal antibodies (MAbs): SDOW17 (National Veterinary Services Laboratories - NVSL, Ames, IA) and SR30 (kindly provided by Dr. Eric Nelson (South Dakota State University, SD - USA). In addition, we also developed a mAb specific to

a 15-mer synthetic peptide corresponding to an immunodominant epitope of PRRSV M protein (de Lima et al., unpublished data). This mAb was used to fine map the core residues of this epitope and examine the specificity of the rescued mutant viruses carrying amino acid substitutions within this region. Preliminary characterization of this mAb demonstrated its ability to recognize MARC145 cells infected with several US-type PRRSV isolates and reference strains. Further analyses also showed its usefulness in specifically recognize PRRSV M protein by Western blot and radioimmunoprecipitation studies (not shown). The secondary antibody used was a goat anti-mouse IgG antibody (Alexa Fluor - 488, Molecular Probes, Eugene, OR).

2.2. Introduction of deletions into the full-length PRRSV cDNA infectious clone

Two regions were previously selected for deletion in each of the following proteins: NSP2, Gp3 and M. Each region spans 15 amino acids residues which were found to be highly immunogenic, conserved among US- and/or European-type PRRSV strains and most importantly, consistently recognized by antibodies of PRRSV-infected pigs [12]. The regions selected as serological marker candidates were (P^{431}PPPPRVQPRKTKSV^{445} and K^{441}TKSVKSLPGNKPVP^{455} within Nsp2; Q^{61}AAAEVYEPGRSLWC^{75} and G^{91}FMVPPGLSSEGHLT^{105} within Gp3 and, L^{151}KSLVLGGRKAVKQG^{165} and A^{161}VKQGVVNLVKYAK^{174} within M protein amino acid sequence). All deletions were introduced into the pFL12 plasmid which contains the full-length cDNA of NVSL 97-7895 PRRSV strain [14], by overlap extension method as previously described [16]. Sequences from either side of the point of deletion were amplified by using primers specific for each protein designed such that their 3'ends hybridize to template sequence on one side of deletion and the 5' ends are complementary to template sequence on the other side of the deletion (see Table 1). Using this approach the products generated from the PCR reaction using the reverse

and forward primers with overhang regions are therefore overlapping at the deletion point. After amplification of the flanking regions, both amplicons were purified from a low melting point gel and precipitated by phenol-chloroform using standard protocol as described elsewhere [17]. Gel-purified DNA were mixed equally and submitted to 4-5 cycles of PCR followed by addition of external primers. After gel purification and precipitation the DNA was digested with *Spe*I and *Sph*I (NSP2), *Bst*BI and *Bsr*GI (ORF3) and *Bst*EII and AflII (ORF6) and cloned directionally into the intermediate vector (EPpBR322) which contains the structural genes and 3`UTR of PRRSV genome. After confirming the deletion and absence of any other mutations within the region digested by specific restriction enzymes by sequencing, the intermediate vector was digested with *Eco*RV and *Pac*I and the fragment was cloned directionally into pFL12.

2.3. In vitro transcription, RNA electroporation and, recovery of epitope deletion mutants

The full-length plasmid (pFL12) was digested with AclI and linearized DNA was used as the template to generate capped RNA using the mMESSAGE mMACHINE Ultra T7 kit according to manufacturer's (Ambion) recommendations. Briefly, after *in vitro* RNA transcription, the reaction mixture was treated with DNaseI to digest the DNA template, extracted with phenol/chloroform and finally precipitated with isopropanol. Following electrophoresis through a glyoxal agarose gel, the integrity of the RNA transcripts was analyzed upon ethidium bromide staining of the gel.

Sub-confluent MARC-145 cells were used for electroporation with approximately 5μg of *in vitro* produced transcripts along with 5μg of carrier RNA isolated from uninfected MARC-145 cells. About $2x10^6$ cells in 400 μl of DMEM containing 1.25% DMSO were pulsed once using Bio-Rad Gene Pulser Xcell at 250V, 950μF in a 4.0 mm cuvette. After, the cells were diluted in normal growth media containing 10% of fetal bovine serum (FBS) and

placed into a 60-mm cell culture plate. The expression of N protein at 24-48 hours post RNA electroporation was interpreted as an indicator of genome replication and transcription. After confirming expression of N protein using indirect immunofluorescence assay (IFA), the culture supernatant from electroporated cells was collected at 48-72 hours post-electroporation, clarified and inoculated onto a confluent monolayer of uninfected MARC-145 cells. The cells were then monitored on a daily basis for characteristic cytopathic effect (CPE) and also examined for expression of N protein. Culture supernatants from infected cells showing both CPE and positive fluorescence were assigned to contain infectious virus. The rescued virus was amplified and small aliquots were stored at -80°C for further studies. In all the experiments, FL12 (containing wild-type PRRSV genome) and FL12 pol^- (containing polymerase-defective PRRSV genome) were used as positive and negative controls respectively, as described elsewhere [14].

2.4. Kinetics of virus growth

MARC-145 cells were infected either with FL12 or FLdNsp2/44 at a MOI of 0.1 $TCID_{50}$ per cell and incubated at 37°C in 5% CO_2 atmosphere. Aliquots of culture supernatants from infected cells were collected at different time points (0, 6, 12, 24, 48 and 72 hours post-infection) and the virus titer was determined by limiting dilution and the titers were expressed as tissue culture infectious dose 50 per ml ($TCID_{50}$/ml). The viral growth kinetics assays was performed in triplicate.

2.5. Animal experiments

Two groups of fifteen mixed-breed (Landrace x Large White) piglets averaging 3 weeks of age obtained from a PRRSV-free farm were allocated in three BL-2 isolation rooms. At the beginning of the experiment, all animals were tested negative for PRRSV-specific

antibodies as measured by a commercially available ELISA kit (IDEXX Labs, Inc). The animals from the experimental groups were inoculated with a total dose of $10^{5.0}$ $TCID_{50}$/3ml of PRRSV FL12 or the epitope deletion mutant (FLdNsp244) by intranasal (1ml in each nostril) and intramuscular (1ml) routes. The inoculated animals were clinically monitored on a daily basis and their rectal temperatures were recorded from day 3 pre-inoculation to day 15 post-infection (pi). Sequential blood samples were collected from all animals at days 0 (zero), 7, 15, 30, 45 and 60 pi. In order to assess the virulence of the epitope deletion mutant (FLdNsp2/44), we inoculated two pregnant sows at day 90 of gestation by intranasally route with $10^{5.0}$ $TCID_{50}$/ml of the mutant virus. The sows were acquired from a specific-pathogen free herd with a certified record of absence of PRRSV infection. Inoculated sows were clinically monitored from 3 days prior to inoculation until 15 days post-farrowing. Blood samples were collected at days 0 (zero), 7, 15, 30, 45 and 60 pi for serological and virological analysis.

2.6. Peptide ELISA

Serum samples collected until day 60 pi from all piglets experimentally infected with FL-12 (wild-type strain) and FLdNsp2/44 (epitope deletion mutant) and from the sows infected with the PRRSV deletion mutant were submitted to a peptide-based ELISA for screening of the peptide-specific antibody response, as previously described [12]. Briefly, Immulon 2HB flat bottom microtiter 96 well plates (Thermo Electron, Milford, MA) were coated with 100µl of a peptide solution (10µg/ml) in 0.1M carbonate buffer (pH 9.6), and incubating overnight at 4ºC. After blocking with 250µl of a 10wt. % nonfat dry milk solution for 4h at room temperature on a plate shaker, the plates were washed three times with PBS containing 0.1% Tween 20 (PBST-20). Unbound reagents were further removed by striking the plates repeatedly, bottom up, on a stack of absorbent paper towel. Then, 100µl of pig sera

(1:20) diluted in 5wt. % nonfat dry milk in PBST-20 was added per well and plates were incubated in the shaker for 1h at room temperature. After washing five times with PBST-20, each well received and was incubated with 100µl of the affinity purified antibody peroxidase labeled goat anti-swine IgG (KPL, Gaithersburg, MD) diluted 1:2000 in PBST-20 with 5wt. % nonfat dry milk for 30min at room temperature. Following a final wash, 100µl of ABTS (KPL) peroxidase substrate was added for 15 min at 37ºC and the reaction was stopped by adding 100µl of SDS 1%. A 15-mer synthetic peptide (ep 44 sequence) which has been shown to be recognized by PRRSV-specific antibodies [18] was used as a positive peptide control. Sera were considered positive when the OD value was above the cutoff point (the mean OD absorbance at 405nm of the negative sera plus 3 standard deviations).

2.7. Bioinformatics analysis

Multiple alignment of nucleotide and amino acid sequences obtained either from Gen Bank or from sequencing facilities were made using ClustalW [19]. Alignments were retrieved and analyzed by Bio-Edit sequence alignment editor v.7.0.5.

3. Results

3.1. Recovery of a mutant PRRSV lacking an immunodominant B-cell epitope of Nsp2

In order to investigate whether an immunodominant region within Nsp2 was dispensable for viral replication, we introduced a deletion of 45 nucleotides into the full-length cDNA infectious clone of PRRSV (FL12). A mutant PRRSV virus named FLdNsp2/44 carrying a deletion of amino acid residues P^{431}PPPPRVQPRKTKSV^{445} was successfully rescued after RNA electroporation into MARC145 cells. Such segment corresponds to an immunodominant epitope localized in a predict hydrophilic domain of Nsp2, and it was found

to be consistently recognized by antibodies from pigs experimentally infected with a North American strain of PRRSV (NVSL97-7895) [12]. Although the presence of the 45 nucleotide deletion introduced into the FLdNsp2/44 mutant had been previously checked by sequencing in each of the cloning steps, it was further confirmed after sequencing of one fragment amplified by RT-PCR directly from the culture supernatant collected from infected MARC 145 cells (not shown).

3.2. In vitro growth kinetics of the epitope deletion mutant (FLdNsp2/44) and its parental virus (FL12)

In order to investigate the possible effects of the deletion of the Nsp2 epitope on virus growth, MARC 145 cells were infected in parallel (MOI of 0.1) with either FL12 or FLdNsp2/44. Aliquots of culture supernatants from infected cells were collected at different time points after infection and the virus titer was determined. The viral growth kinetics assays was performed in triplicate and the mean titer values obtained in each time point can be observed in Figure 1. The multiple step growth curves revealed that the mutant virus exhibited a delayed onset of extracellular virus accumulation, reaching its maximal titer approximately 24 hours later than the parental virus. Although the titer reached by the mutant virus was lower than that of the wild type, similar titers were obtained for both viruses in subsequent passages in cell culture (not shown). These results demonstrate that the removal of the Nsp2 B-cell epitope did not affect the efficiency of replication *in vitro* of the mutant virus.

3.3. Serological response to the mutant virus assessed in weaned piglets

The serological response to the mutant virus was assessed by inoculation of two groups of fifteen piglets with a total dose of $10^{5.0}$ $TCID_{50}$ either of FL12 (PRRS virus recovered from the NVSL 97-7895 strain full-length cDNA infectious clone) or FLdNsp2/44

by intranasal and intramuscular routes. Piglets inoculated with FL12 were considered the positive control group. No significant increase in rectal temperature was observed among the inoculated animals after the virus inoculation, except for a slight increase in rectal temperature (≤ 1.5ºC) between days 2 and 5 post-infection (pi) in the group infected with the wild-type virus (FL12). In both experimental groups, viremia was detected from days 4 to 15 pi with a peak in viral titers on day 7 pi ($10^{3.32}$ ±0.22 for FLdNsp2/44 and $10^{4.28}$ ±0.49 $TCID_{50}$/ml for FL12) in the serum samples indicating active viral replication (Table 2). Sera collected from the experimentally infected animals in both groups through days 15 to 60 were positive for PRRSV-specific antibodies as assayed by a commercially available ELISA kit (Idexx Labs, Inc) (Figure 2).

A 1076bp genomic fragment was amplified by RT-PCR from virus isolated from sera of 5 infected piglets at day 10 pi and sequenced, confirming the identity of the epitope deletion mutant. The results of virus isolation from blood and subsequent positive serology demonstrated that a productive virus replication occurred in all experimentally infected piglets. These data indicates that the epitope deletion did not affect negatively the whole humoral immune response elicited by the mutant virus. In contrast, pigs inoculated with the epitope deletion mutant (FLdNsp2/44) completely lacked antibodies against the peptide (ep#44) corresponding to the deleted epitope, whereas a strong reactivity was observed in the sera obtained from animals infected with the wild-type virus (FL12) (Figure 3).

3.4. Assessment of virulence of the PRRSV epitope deletion mutant in a pregnant sow model

In order to study the properties of the epitope deletion mutant (FLdNsp2/44) *in vivo*, we used a pregnant sow model which has been extensively used in our lab to assess virulence of wild type and mutant viruses [15, 17]. We evaluated the phenotype of the mutant virus in this reproductive failure model according to the viability of the offspring at birth and upon

weaning at 15 days after farrowing. The virulent phenotype of FL12 was unequivocally demonstrated by the high rate of mortality at birth (Table 3). Likewise, the epitope deletion mutant (FLdNsp2/44) was invariably virulent when inoculated in pregnant sows inducing abortion rates comparable to its parental virus (Table 3). In one sow, abortion was observed 10 days after infection with FLdNsp2/44 and presence of the virus was confirmed by real-time PCR from RNA extracted from thoracic fluid and tissues collected from aborted fetuses (data not shown). As shown in Table 3, two piglets born from one sow infected with the mutant virus were alive at 15 days post-farrowing; however, they were euthanized due to weakness and poor health condition. These results show that deletion of 15-mer Nsp2 epitope did not contribute for attenuation of the virus in the pregnant sow model.

3.5. Glycoprotein 3 and M protein immunodominant epitopes are not dispensable for virus growth

Deletion of previously identified epitopes, selected as serological markers candidates, from Gp3 and M protein were found to be lethal for virus recovery. Three different clones of each construct were tested by electroporation into MARC145 cells with full-length RNA produced *in vitro*, and all attempts to recover infectious virus were unsuccessful. The possibility of additional mutations or frame change due to DNA manipulation in regions other than the selected target was discarded after sequencing of the entire cloned fragment. Thus, we attribute the lack of replication to the effects of the introduced deletions, being plausible to assume that the selected epitopes are somehow essential for virus replication *in vitro*. As removal of the entire epitopes (15aa) was deleterious for virus replication, further attempts aimed to eliminate the immunogenicity of the epitope 201 (highest level of amino acid conservation among the candidates tested) without impairing the structure and/or function of the protein were carried out. To this purpose, we generated three additional constructs in

which each five amino acids were altered to alanine in the five first amino acid stretch (FL-201SubAVKQG), in the next five amino acids (FL-201SubVVNLV) or in the last four residues (FL-201SubKYAK) of the selected region. Among the three mutants generated, only one virus (FL-201SubKYAK) was successfully rescued after RNA electroporation. This virus replicated to a low titer and with a slightly delayed cell-to-cell spread observed after passage of the culture supernatant onto a naive monolayer of MARC145 cells (data not shown). The presence of the specific amino acid substitutions and absence of any other additional mutations were confirmed by nucleotide sequencing. MARC145 cells infected with FL-201SubKYAK was recognized by the monoclonal antibody SR30 (directed to an N protein epitope) and also by a monoclonal antibody developed against the peptide (from M protein) corresponding specifically to the selected epitope. Thus, the amino acid changes of the last four residues still resulted in a marker positive virus indicating that most likely the core residues recognized by antibodies are located within the first 10 amino acid stretch. Based on these findings, we decided not to use this mutant virus for animal experiments since the amino acid substitutions within the selected epitope could not completely abolish its immunogenicity.

4. Discussion

The use of marker vaccines accompanied with its corresponding differential diagnostic test have become popular and even mandatory in actions aiming the control and eradication of animal diseases with economic impact for national and international trade [8,20]. The best examples of eradication programs successfully achieved by the use of marker vaccines have been previously demonstrated for important livestock diseases such pseudorabies and infectious bovine rhinotracheitis [8]. The development of the marker vaccine for these dsDNA

viruses consisted in the deletion of a gene encoding an immunogenic, conserved protein and dispensable for viral replication, without impairing the whole immunogenicity of the vaccine virus [8]. Other examples of marker vaccines include licensed subunit vaccines against classical swine fever (CSF) based on the recombinant expression of the E2 of CSF virus in a baculovirus system [21] and its differential serological test [22]. However, it is generally accepted that there is still need of improvement regarding the efficacy of these new subunit vaccines when compared to the classical live-attenuated CSFV vaccine [23,24]. Promising results have also been described for another flavivirus, the bovine viral diarrhea virus, by using a similar methodology as described for CSFV despite the incomplete protection conferred against a wide-range of BVDV isolates [25,26]. Further approaches aiming the development of a marker vaccine have also been reported for Newcastle disease virus (NDV) either by construction of recombinant chimeric viruses [27], or deletion of immunodominant epitopes [28].

Vaccination against PRRSV infections is being carried out in the United States since 1995. The most commonly vaccine currently in use contains a North American PRRSV strain attenuated by multiple passages in cell culture. Modified-live vaccines are often preferred than inactivated-virus formulations due to its higher efficacy and long-lasting immunity induced. Despite the availability and extensive use of live-attenuated PRRSV vaccines in the United States, an important drawback of these vaccines consists in the lack of a marker feature [12]. Control and eventual eradication of PRRSV infections could be more easily achieved by a systematic vaccination program with a new generation of DIVA vaccines accompanied with a differential diagnostic test which allows serological discrimination between vaccinated and naturally infected animals. This technical improvement on the current attenuated PRRSV vaccines would be an advance for PRRSV vaccinology and consequently highly desirable in eradication programs.

Using a systematic and detailed approach, we previously demonstrated by Pepscan analysis the presence of B-cell linear epitopes in the Nsp2 and structural proteins of a North American strain of PRRSV, consistently recognized by the humoral immune response elicited by PRRSV-infected pigs [12]. In this context, the selection of immunodominant epitopes and deletion of these regions in a full-length infectious cDNA clone would be an alternative approach for the development of marker live-attenuated PRRSV vaccines since the genome of small RNA viruses, like PRRSV, generally does not tolerate subtle changes such as deletions of entire genes [9-11].

We herein describe the generation of a PRRSV carrying a deletion of an immunodominant B-cell linear epitope of Nsp2 previously identified to be recognized by antibodies from PRRSV-infected animals [12]. The successful recovery of the epitope deletion mutant (FLdNsp2/44) and its active replication in pigs, demonstrated that this specific region is dispensable for virus replication *in vitro* and *in vivo*. Previous reports have shown that the Nsp2 replicase protein of PRRSV does tolerate insertions [29], and deletions [30], representing an ideal target for the development of marker vaccines [31]. The rescued mutant virus also exhibited similar growth kinetics *in vitro* when compared to the wild type virus despite the lower titers reached in the first passages in cell culture (Figure 1). In addition, the duration of viremia in piglets upon experimental inoculation was comparable to the parental virus, although lower titers have been detected in the sera of the infected animals through days 4 to 15 post-inoculation (Table 2). A fragment of 1076 bp encompassing the deleted region within Nsp2 was amplified by RT-PCR from virus isolated from sera of five infected piglets at day 10 pi. Nucleotide sequencing of the fragment confirmed the identity of the epitope deletion mutant in all five samples indicating that the deletion was stable after several rounds of replication *in vivo*.

We further examined the *in vivo* phenotype of the epitope deletion mutant by inoculating two pregnant sows at day 90 of gestation. Infection of pregnant sows with virulent PRRSV strains invariably results in abortion, mummified or stillbirth piglets being a reproductive failure model to study virulence. Infection of the pregnant sows with FLdNsp2/44 resulted in abortion in similar levels when compared to the wild type virus indicating that the removal of the 45 nucleotides from Nsp2 did not result in attenuation. Although the absence of virulence is an essential requirement for any live virus vaccine, we did not expect that any level of attenuation could be attributed to the 15 amino acid deletion. Furthermore, the attenuation property of the mutant virus was not a major focus of our study. Previous findings in our lab have been indicated that the determinants of virulence are located in regions of the PRRSV genome other than Nsp2 [15].

On the other hand, removal of B-cell epitopes corresponding to the previously identified serological marker candidates (see material and methods) within Gp3 and M protein were lethal for virus recovery. This could unequivocally be attributed to the deletion of the 15 aa since the entire cloned fragment was sequenced to exclude the possibility of any additional mutation introduced during the cloning steps. However, as the amino acid residues A^{161}VKQGVVNLVKYAK^{174} within the M protein correspond to an immunogenic segment with the highest level of amino acid conservation among our marker candidates, further attempts were made in order to obtain a mutant virus deprived of the immunogenicity of this region.

Hypothesizing that the complete removal of this B-cell epitope could somehow alter the structure and/or function of the M protein, we alternatively constructed three different viruses carrying amino acid substitutions within this region. Such approach might eliminate the immunogenicity of the region without impairing structure/function of the protein and consequently allow virus replication *in vitro*. Only one of the new constructs with amino acid

changes in the four last residues of the epitope was successfully rescued from RNA-transfected MARC 145 cells. The epitope was still recognized by a mAb specifically produced against the 14-mer peptide corresponding to the epitope indicating that the core residues are located within the first 10 aa stretch (not shown). Eventually, the first five amino acids ($A^{161}VKQG^{165}$) could be considered the minimal sequence recognized by antibodies since this segment corresponds to the overlapping residues previously found to be reactive with antibodies by a Pepscan mapping [12]. Further studies should focus on identifying the minimal region associated with immunogenicity and introduce point mutations to eliminate it from the selected epitope.

In summary, *in vitro* and *in vivo* characterization of the FLdNsp2/44 showed that removal of a 15-mer Nsp2 epitope had no effect on immunogenicity, growth properties or virulence of the mutant virus. In addition, pigs inoculated with FLdNsp2/44 did not develop antibodies to the selected epitope as measured by a peptide-based ELISA, whereas a strong reactivity was observed in the sera derived from animals infected with the wild-type virus (FL12).

Taken together, our results provide proof of concept demonstrating the feasibility of constructing a PRRSV live-attenuated marker vaccine by deleting an immunodominant B-cell linear epitope. In addition, the combination of a mutant virus carrying an epitope deletion and its corresponding peptide-based ELISA represents an attractive approach for the development of PRRS differential vaccines. Efforts will now focus on identifying an optimal marker or a combination of peptide markers capable of reliably inducing a consistent serologic response in the diverse universe of wild type PRRSV strains.

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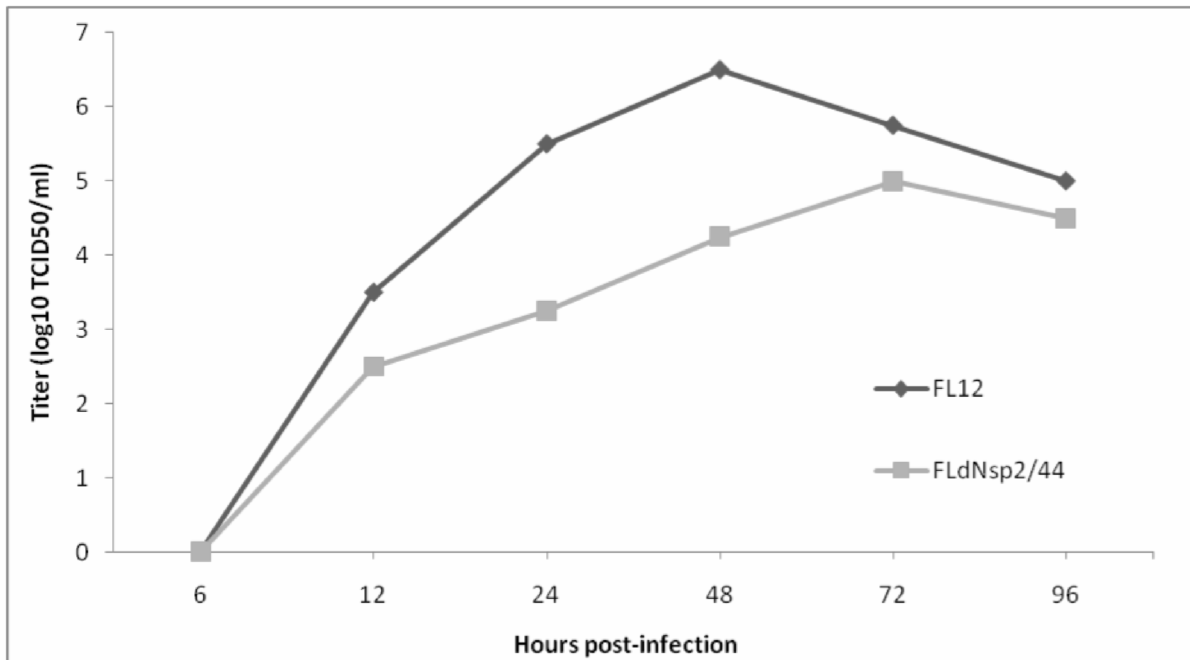

Figure 1. Growth kinetics of the epitope deletion mutant (FLdNsp2/44) and its parental virus (FL12). MARC145 cells were infected at a MOI of 0.1 and culture supernatant was collected at different time points after infection. Virus infectivity was quantitated by limiting dilution and virus titers were calculated according to Reed and Müench method. Results represent the mean values obtained in three independent experiments.

Table 1. Primers used for amplification of specific fragments in each of the selected targets for deletion. Primers were designed such that their 3'ends hybridize to template sequence on one side of deletion and the 5' ends are complementary to template sequence on the other side of the deletion. The selected protein and primers with its 5'-3' nucleotide sequence are shown.

Protein	Primer name	Nucleotide sequence (5' – 3')
ORF6	d200Rev	CAAGGTTTACCACCCCGGGCACCAATG
ORF6	d200For	CATTGGTGCCCGGGGTGGTAAACCTTGTCAAATATG
ORF6	d201Rev	GGCATATTATTTTCTGCCACCCAACACGAGGCTTTTC
ORF6	d201For	GTGGCAGAAAAtAATATGCCAAATAACAACGGCAAGC
ORF3	d129Rev	CATCGGTCATGCTCAGCGGCTGCTTGCCGGGTG
ORF3	d129For	GCCGCTGAGCATGACCGATGTAGTGAGGACG
ORF3	d132Rev	GCGTAAACACTTAGATCGTCATGGTCGTCCTC
ORF3	d132For	CATGACGATCTAAGTGTTTACGCCTGGTTGGCG
NSP2	d44Rev	GTTCCCTGGCAAGCTCTTCGGTGTCCACCGTGG
NSP2	d44For	CCCACGGTGGACACCGAAGAGCTTGCCAGGGAAC
NSP2	d45Rev	CTGACCTTCCTGCGTGGAGCTCGAGGCTGAACTCTTGG
NSP2	d45For	CCAAGAGTTCAGCCTCGAGCTCCACGCAGGAAGGTCAG

Table 2. Viremia in 15 pigs experimentally infected with 10^5TCID$_{50}$/ml of FL12 (wild type) or FLdNsp2/44 (epitope deletion mutant). Infectivity is expressed as mean log$_{10}$ PRRSV titer TCID$_{50}$/ml^{-1} in the sera of the 15 experimentally infected pigs from days 4 to 30 post-infection ± the standard deviation.

Group	4dpi	7dpi	10dpi	15dpi	30dpi
FLdNsp2/44	2.88 (±0.38)	3.32 (±0.22)	2.55 (±0.46)	2.29 (±0.42)[1]	ND[2]
FL12	3.86 (±0.43)	4.28 (±0.49)	4.25 (±0.38)	3.41 (±0.20)	ND

[1] 13 out of 15 pigs showed detectable levels of virus in sera
[2] ND=Not detectable (< $10^{1.7}$ TCID$_{50}$/ml)

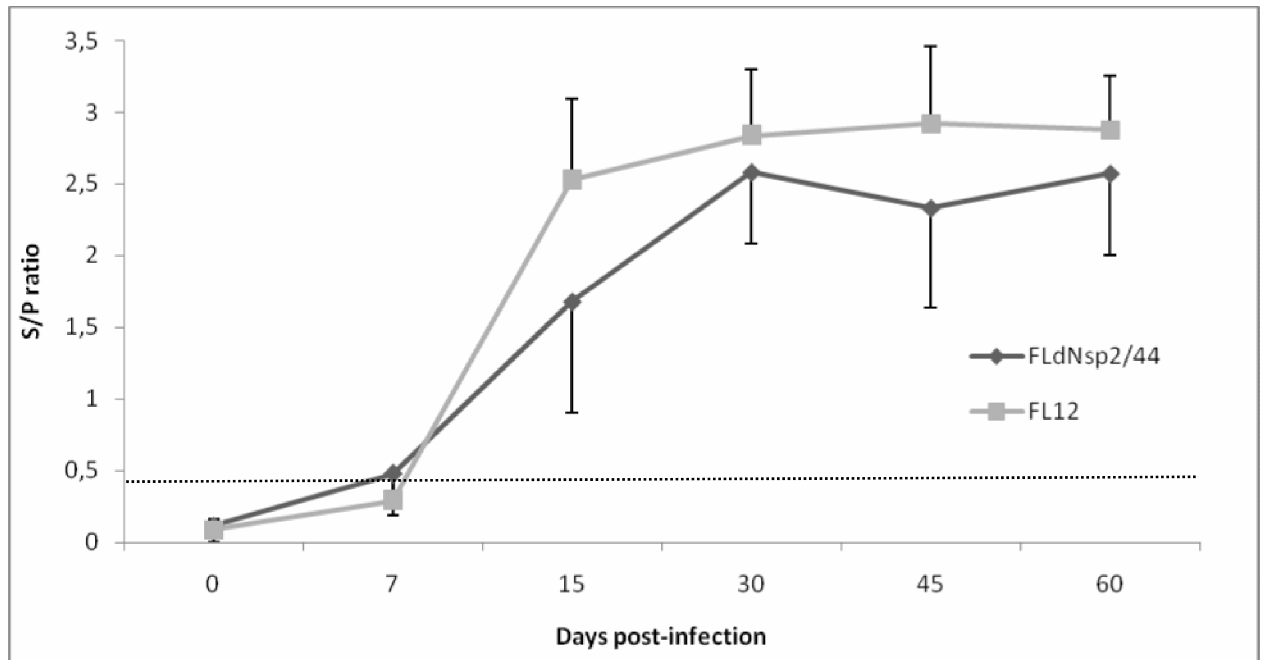

Figure 2. Serum antibody titers in piglets experimentally inoculated with PRRSV FL12 strain (wild type virus) or FLdNsp2/44 (epitope deletion mutant). S/P ratios were expressed according to a commercial IDEXX ELISA kit and the mean values obtained from the serum samples collected at 0, 7, 15, 30, 45 and 60 days post-infection from the 15 piglets infected in each group are shown. Vertical bars represent the standard deviation. A dashed line at 0.4 S/P ratio corresponds to the threshold value above which samples are considered positive for PRRSV-specific antibodies.

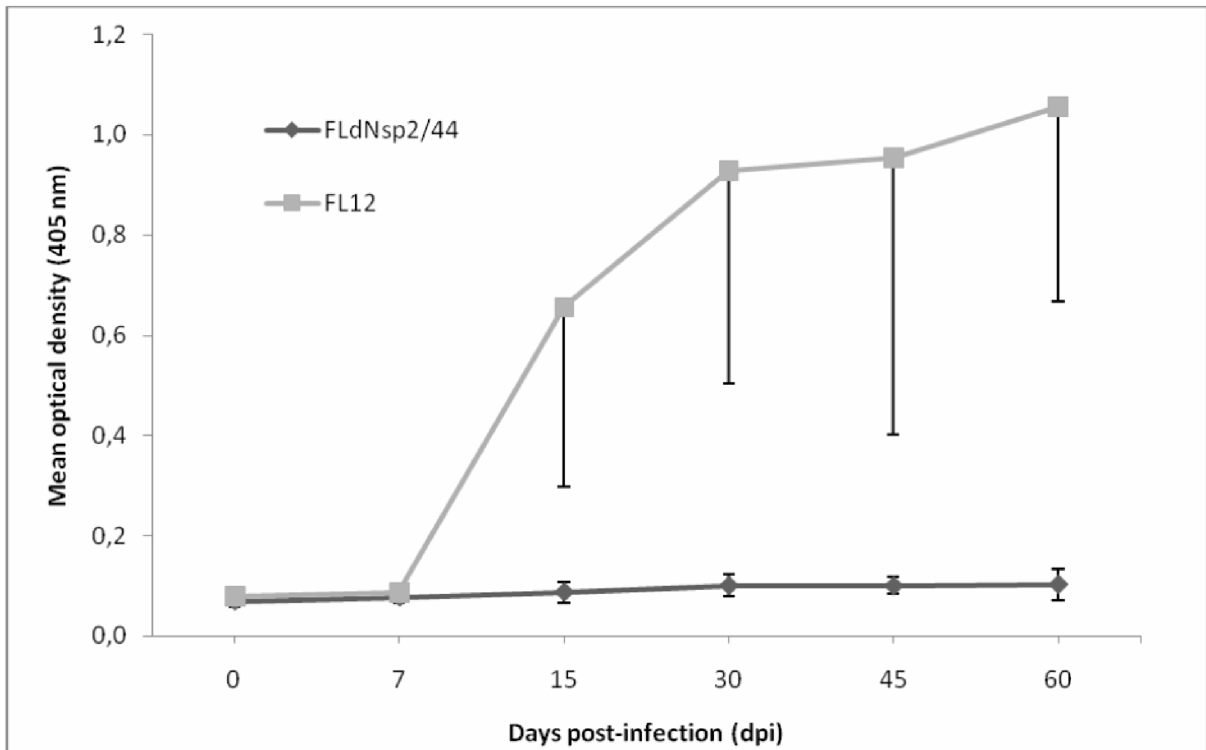

Figure 3. Serological response of pigs experimentally infected with $10^5 TCID_{50}$/ml of FL12 (wild type) or FLdNsp2/44 (epitope deletion mutant) from day 0 to 60 post-infection. The values represent the mean optical density obtained in a peptide-based ELISA where plates were coated with a 15-mer peptide corresponding to the deleted region in FLdNsp2/44. Vertical bars indicate the standard deviation.

Table 3. *In vivo* phenotype of the wild-type virus (FL12) and epitope deletion mutant (FLdNsp2/44) assessed in a reproductive failure model in pregnant sows (90 days of gestation). The viability scores of offspring at birth and 15 days after farrowing are indicated.

Group	Sow #	Offspring	Viability at birth		Viability at 15 days
			dead	live	live
FLdNsp2/44	1	18	18	0	0
	2	14	11	3	2
FL12[1]	1	16	13	3	0
	2	14	13	1	0

[1] Data obtained from a previous experiment (Kwon et al., 2006)

5. CONSIDERAÇÕES FINAIS

A cadeia produtiva de suínos no Brasil tem apresentado um crescimento expressivo da produção e das exportações de carne nos últimos anos. A indústria nacional possui um sistema de produção altamente tecnificado, sendo comparável ao empregado em países desenvolvidos e produtores tradicionais. Apesar de o Brasil ser considerado área livre do vírus da síndrome respiratória e reprodutiva dos suínos (PRRSV), é imperativo um monitoramento contínuo dos rebanhos visando à manutenção deste *status*. Desta forma, o grande desafio imposto aos países onde a suinocultura é expressiva inclui as questões referentes ao diagnóstico, profilaxia e controle das enfermidades que causam um impacto negativo a produção. Nesse sentido, é essencial um conhecimento detalhado de enfermidades, como a PRRS, que são associadas a problemas essencialmente reprodutivos causando um impacto econômico significativo à suinocultura.

Os estudos apresentados na presente tese abordam aspectos referentes ao desenvolvimento de uma nova geração de vacinas atenuadas para serem usadas na profilaxia, controle e em eventuais programas de erradicação do PRRSV. Mais especificamente, estão descritos experimentos onde foram identificados vários segmentos de diferentes proteínas como candidatos a marcadores sorológicos e uma avaliação da dispensibilidade destas regiões a replicação viral *in vitro* e *in vivo*. Além disso, avaliou-se a resposta sorológica induzida por animais infectados com um vírus carreando a deleção de uma região-alvo previamente selecionada.

No primeiro experimento (Capítulo 2), estão apresentados os resultados de um mapeamento de epítopos lineares de linfócitos B por *Pepscan* nas diferentes proteínas do PRRSV. Resumidamente, avaliou-se a reatividade do soro de 15 suínos infectados com uma cepa norte-americana do PRRSV com peptídeos sintéticos correspondentes a seqüência completa de aminoácidos da proteína não-estrutural 2 (Nsp2) e de todas as proteínas estruturais do vírus. Os resultados demonstram a identificação de vários segmentos imunogênicos localizados nas regiões mais hidrofílicas das proteínas analisadas. As regiões preferencialmente reconhecidas pelo soro da maioria dos animais (imunodominantes) e relativamente conservadas entre diferentes cepas/isolados do PRRSV foram selecionadas como candidatas a marcadores sorológicos.

Considerando-se que diversos estudos têm demonstrado que o genoma do PRRSV não tolera a deleção completa ou de grandes segmentos da maioria dos genes, os resultados

descritos no Capítulo 2 representam o primeiro passo no que se refere à identificação de possíveis marcadores sorológicos. Ou seja, pequenos segmentos conservados e immunodominantes de diferentes proteínas seriam, eventualmente, dispensáveis a replicação viral, constituindo-se assim em marcadores sorológicos negativos. Isto representaria um avanço no que se refere à elaboração de uma nova geração de vacinas contra o PRRSV, uma vez que vacinas contendo estes marcadores sorológicos negativos induziriam uma resposta sorológica capaz de ser detectada por um teste sorológico diferencial.

Nos experimentos descritos no Capítulo 3, foram deletados segmentos genômicos correspondentes a alguns epítopos previamente selecionados como candidatos a marcadores sorológicos. Essas regiões correspondiam a epítopos imunodominantes e conservados identificados na proteína não-estrutural 2 (Nsp2), glicoproteína 3 (Gp3) e proteína M. Todas as deleções foram introduzidas em um clone infeccioso de cDNA visando avaliar a dispensabilidade destas regiões para a replicação viral *in vitro* e *in vivo*.

Os resultados referentes a esta segunda parte do estudo demonstram a produção de vírus mutantes carreando deleções de epítopos imunodominantes e relativamente conservados da Nsp2. A caracterização de um dos vírus mutantes (FLdNsp2/44) indicou que a deleção da região genômica correspondente ao epítopo não afetou negativamente a replicação viral *in vitro*. Além disso, o vírus replicou eficientemente em animais, induzindo uma resposta sorológica com nível e duração comparáveis àquela induzida pela infecção com o vírus parental. Mais importante, foi possível a discriminação sorológica entre animais infectados com o vírus mutante e com o vírus parental por um teste de ELISA contendo como antígeno o peptídeo sintético correspondente ao epítopo deletado.

Em resumo, os resultados referentes aos Capítulos 2 e 3 demonstram a viabilidade de produção de uma vacina atenuada diferencial para o PRRSV pelo uso da metodologia apresentada. Embora os dados obtidos sejam provenientes de grupos de animais experimentalmente infectados, ficou comprovada a possibilidade de diferenciação sorológica com base na reatividade de anticorpos específicos com um peptídeo correspondente a um epítopo linear de linfócitos B identificado na Nsp2. Estudos subseqüentes devem se concentrar na identificação de marcadores sorológicos adicionais que sejam reconhecidos pela resposta humoral de animais infectados com diversos isolados de campo do PRRSV.

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