RESEARCH REPORT
SWINE HEALTH

Title: Development of a modified live vaccine against PRRSV with optimal DIVA marker potential" project - **NPB#08-248**

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Date Submitted: August 2, 2010

Keywords: PRRSV, DIVA Vaccine, Live Vaccines, Epitopes, Marker Vaccine

Industry Summary

Work in our laboratories is exclusively oriented towards the development of a new generation of PRRSV vaccines that would confer broad protection. We work, through different basic and applied projects and with the participation of different members of our laboratories, towards such main goal. This particular proposal has been aimed **at developing an optimal marker differential vaccine system for the new generation of vaccines currently under development**. The main hypothesis is that the optimal new generation PRRSV vaccine will be of the live-attenuated type. The live-attenuated PRRSV vaccines are more effective because their components or antigens that are determinants of protection are "seen" by the pig's immune system in a similar way as are seen those of live wild-type (fully infectious) PRRSV. Our ultimate goal is to develop a live vaccine of high safety and efficacy that would be compatible with the ability of cleansing the PRRSV infection from a herd, that is, compatible with the ability of differentiating, through a simple test, the vaccinated/protected animals from those that have suffered infection by wild-type PRRSV. Engineering of new live-attenuated PRRSV marker vaccines requires knowledge of the genetic make-up of PRRS antigens and identifying small areas of the proteins which can be eliminated from the vaccine without affecting the virus' ability to multiply in cells and in the pig. This concept is similar to that successfully applied for the development of Pseudorabies marker vaccines. The differential vaccines, which, like in the example of Pseudorabies, were originally called "marker vaccines" are now also identified as DIVA vaccines (which stands for "Differentiating Infected from Vaccinated Animals"). With previous support from swine producers (NPB 06-177), we had developed the first prototype of DIVA live vaccine for PRRSV through the elimination of small protein fragment (epitope) from the make-up of these vaccines. Such vaccine candidate, although falling short of being a perfect marker vaccine, served as proof of the concept and encouraged further research on more efficient small protein fragments that can be used as markers. We attempted such task through this most recent project now being reported **(#08-248).** The specific objectives of this proposal were: 1) **To develop**

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

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a live PPRSV mutant deprived of the 201 ORF6 epitope reactivity, a small part of the PRRSV M protein which our results would suggest to be the ideal marker, based on its level of conservancy among many PRRSV strains 2) **Testing of this epitope 201-deprived mutant in vivo** , using an standard experimental design for animal inoculation which has been previously tested and described. **3) Field testing of the companion peptide-ELISA specific for the marker epitope**, validating its specificity and sensitivity based on the analysis of a large number of field serum samples. At the end of this NPB supported project we know much more about all these three points, and significant advances have been made, although a final product is not available yet. Thanks to the work conducted under this project, we have now a much better sense of the technical modifications and new constructs that are needed to be explored in order to secure a stable and effective prototype of live marker vaccine. This research is being continued in our laboratories beyond the termination of this project NPB #08-248. A major obstacle to overcome in the next series of experiments has to do with developing a stable live mutant of PRRSV that would not revert to the wild-type type PRRSV after injected in a pig. Such stability of the strain in vivo is essential to maintain the "marker negative" character of the DIVA vaccine strain. The specific points of this research are presented in more detail under the discussion section of this report.

Scientific Abstract

This proposal has been aimed at developing an optimal marker differential vaccine system for the new generation of vaccines currently under development. The central notion is that the optimal *new generation* PRRSV vaccine will be of the live attenuated type. Previous research in our laboratory has indicated that one specific epitope located on ORF6 gene (epitope contained in peptide 201) would be highly conserved in the vast majority of PRRSV type II strains and would then constitute an ideal candidate as a marker for serologic differentiation of infected and vaccinated animals (DIVA principle). Our previous results had indicated that the totality of infected pigs (15/15) mounted antibody response to an epitope in peptide 201 suggesting that this epitope is highly immunogenic. Monoclonal antibody against the epitope in peptide 201 (MAB 201) was then developed. Genomic alignment indicated great level of conservation amongst PRRSV type II strains. Our initial attempts (using reverse genetics) at deleting such epitope (pep 201) from the PPRSV genome ended up in failure. We were able to rescue only one viable deletion mutant which still maintained the immunogenicity corresponding to epitope peptide 201. The specific objectives of this proposal #08-248 have then been : 1) to develop a live amino-acid substitution mutant (rather than a deletion mutant) deprived of the 201 ORF6 epitope reactivity, an epitope which we consider the ideal marker, based on its level of conservancy among strains and its immunodominance, 2) Testing of this epitope 201-deprived mutant in vivo , using an standard experimental design for animal inoculation which has been previously tested and described 3) Field testing of the companion peptide-ELISA specific for the marker epitope, validating its specificity and sensitivity based on the analysis a large number of field serum samples.
Overall, we indentified a highly conserved and immunogenic epitope (designated as epitope 201) in the M protein of type-II PRRSV which can be used as marker for vaccine. We confirmed such conserved character by immunofluorescent analysis of a large number of field isolates, assessed for reactivity with MAB 201 A large majority of field isolates (91 %) was positive for MAB 201 reactivity. Using aa substitution we were able to generate 2 different mutant viruses which harbour a disrupted epitope 201. However, both of these mutants (table 1) were not stable in infected pigs, reverting to wt type after infection of pigs, as early as 7 days pi, as detected in viremic samples collected post-inoculation. These experiments have helped to pinpoint in a more exact manner, the aa residues that are involved in the MAB 201 reactivity. Likewise, the frank back mutations of these constructs obviously indicate a strong selection pressure for wt. As a continuation of these experiments, but beyond the termination of NPB #08-248, we are pursuing an alternative approach. We are now working in constructing mutants that mimic the exact epitopic 201 structure in one of those isolates (~ 9 % of total isolates analyzed) that are naturally occurring with a negative phenotype for epitope peptide 201.We hypothesize that by mimicking the amino acid sequence of a 201–negative field isolate, we can generate a virus that is stable in infected pigs, thus inducing a permanent marker negative serologic profile.

Introduction

Vaccination against PRRSV infections is being carried out in the United States since 1995. The most commonly used vaccine consists of a North American PRRSV strain attenuated by multiple passages in cell cultures. The attenuated vaccines are accepted as the most efficacious vaccines that are currently in the market. Live attenuated vaccines have the ability to induce a solid protective immunity against strains that are closely related to that used to formulate the vaccine but with a variable efficacy against strains that are divergent from the master strain of the vaccine. Because of these current vaccines' variable ability at inducing heterologous protection and their total inability at differentiating vaccinated from infected animals, there is a compelling 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 impose 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). 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, all of them with essential functions, seems to be a more difficult task. Thus, the generation of a mutant virus carrying a deletion of an immuno-dominant 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. Thanks to previous NPB support, 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. Based primarily on the immunodominance and level of amino acid conservation observed for some of the peptides distributed along the different proteins, we selected target epitopes (serological markers candidates) to be deleted in the wild-type infectious cDNA clone (FL12) by reverse genetics. The approach of epitope deletion has proved feasible for arteriviruses (group in which PRRSV belongs), 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. Furthermore, a peptide ELISA based on this particular domain enabled serological discrimination between vaccinated and wild-type virus-infected animals. In our previously funded project, we attempted, using reverse genetics technology, to create deletion mutants that would lack the sequence coding for the marker epitope that we selected on M protein of PRRSV. However, all the deletion mutants we attempted , but one, were not viable, suggesting the importance that these conserved sequences in M protein may have for a fully functional PRRSV. Furthermore, the only viable deletion we produced proved did retain the epitope 201 reactivity, thus invalidating its usefulness as a marker vaccine strain. Based on these previous works , we undertook, under the herein reported NPB ,#08-248, a series of experiments that we describe in the following sections.

Objectives

Objective 1: Developing a live amino-acid substitution mutant deprived of epitope 201-ORF6 antigenic reactivity

Objective 2: In vivo testing of the viable mutant that lacks ep 201-ORF6 antigenic reactivity

Objective 3: Field testing of the peptide ELISA specific for the 201 marker epitope

Materials & Methods

Basic reverse genetics and site-directed mutagenesis

All our experiments have been based on mutants constructed using the full-length PRRSV infectious cDNA clone (FL-12) in pBR322 , an infectious clone that was developed in our laboratories and that has been consistently used since its development in 2003. The infectious cDNA clone (FL-12) was digested with EcoRV and BstZ17I restriction enzymes and the 4.9-kbp fragment encompassing the majority of ORF2, complete ORFs 3 to 7, and the entire3' untranslated region (UTR) of PRRSV was cloned into pBR322 using the same enzyme sites. This intermediate plasmid served as the template for mutagenesis to introduce mutations (adenine substitutions) at the sites shown in table 1 site within M. Mutagenesis was carried out using overlap extension PCR with synthetic primers , Pfu polymerase (Stratagene), and standard techniques. The PCR product was digested with BsrGI and BstEII restriction enzymes and replaced back in the intermediate plasmid. Clones containing the desired mutations were identified and confirmed by sequencing. The entire coding region of M (ORF6) was sequenced to ensure that additional mutations were not present in the clones. The EcoRV-PacI fragment from the intermediate plasmid containing mutations in the M coding region was moved back into the full-length cDNA clone using the same restriction enzyme sites. The M coding region in the full-length clones was again sequenced with PRRSV-specific internal primers to confirm the presence of the mutations.

In vitro transcription, RNA electroporation, and recovery of epitope 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 monolayers of MARC-145 cells were used for electroporation with approximately 5 microgr of in vitro produced transcripts along with 5microg of carrier RNA isolated from uninfected MARC-145 cells. About $2x10^6$ cells in 400 microl of DMEM containing 1.25% DMSO were pulsed once using Bio-Rad Gene Pulser Xcell at 250V, 950 F in a 4.0 mm cuvette. After this treatment, 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 hours post RNA electroporation was 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 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 assessed as containing 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 .

PRRSV(M) Peptide 201- specific ELISA

168 field serum samples collected from seropositive herds through the Veterinary Diagnostic Center (University of Nebraska-Lincoln) were used. Likewise, serums collected from pigs inoculated with the mutants constructed (days 7 to 60 pi) from all piglets experimentally infected with FL-12 (wild-type strain) and each of the epitope 201 M PRRSV-negative mutants (table 1) were tested using a peptide-based ELISA for screening of the peptide 201-specific antibody response. Briefly, Immulon 2HB flat bottom microtiter 96 well plates

(Thermo Electron, Milford, MA) were coated with 100µl of peptide 20 [^{160}KAVKQGVVNLVKYAK^{174}wt 201 sequence, see table 1] solution (10µg/ml) in 0.1M carbonate buffer (pH 9.6), and incubated 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 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 and the plate was incubated 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 1% SDS. 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).

Results

Objective 1: Developing a live amino-acid substitution mutant deprived of epitope 201-ORF6 antigenic reactivity

First, we sought to determine the minimal amino acid residues that are critical for the antigenicity of epitope 201. We cloned entire ORF6 of a PRRSV strain FL12 into an expression vector under a CMV promoter. After that, different mutation combinations were generated (table 1).

Table 1: Scanning for amino acid residues that are critical for the antigenicity of epitope 201

The constructs were then transfected into BHK21 cells and the antigenicity was examined by indirect immune-fluorescent assay using anti-201 MAb. We found that the last 4 a.a are not involved in binding to anti-201 MAb. All other mutation combinations result in disruption of epitope 201.

Constructs	Amino acid sequence	Recognition by anti-201 MAb	Virus recovery
Wt	^{160}KAVKQGVVNLVKYAK^{174}	-	-
A160-163	^{160}AAAAQGVVNLVKYAK^{174}	-	-
A164-167	^{160}KAVKAAAANLVKYAK^{174}	-	-
A168-170	^{160}KAVKQGVVAAAKYAK^{174}	-	+
A171-174	^{160}KAVKQGVVNLVAAAA^{174}	+	ND
PHOBIC 1	^{160}AAVAAGVVNLVKYAK^{174}	-	-
PHOBIC 2	^{160}KAVKQGVVALVAYAA^{174}	-	-
N168R	^{160}KAVKQGVVRLVKYAK^{174}	-	+
*determine by indirect immuno-fluorescent staining using anti-201MA ND: not done			

Next, we introduced these mutation combinations into FL12 cDNA infectious clone. Among 6 mutation combinations, we were able to recover virus from only 2 constructs including N168 R and A168-170 (table 1). As expected,

both of these viruses were not recognized by anti-201 MAb. We sought to determine the minimal amino acid residues that are critical for the antigenicity of epitope 201. We cloned entire ORF6 of a PRRSV strain FL12 into an expression vector under a CMV promoter. After that, different mutation combinations were generated (table 1). The constructs were then transfected into BHK21 cells and the antigenicity was examined by indirect immune-fluorescent assay using anti-201 MAb. We found that the last 4 a.a are not involved in binding to anti-201 MAb. All other mutation combinations result in disruption of epitope 201.

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Objective 2: In vivo testing of the viable mutant that lacks ep 201-ORF6 antigenic reactivity

Four weaned pigs were infected with N168R and another 2 were infected with A168-170. Serum samples were collected periodically after infection. Serum samples were inoculated onto MARC145 cells. The cells were then immuno-stained with anti-201 MAb. All serum samples at 7 days PI contained viruses that are recognized by anti-201 MAb (Fig.1). Entire ORF6 of the viruses from serum samples was RT-PCR amplified and sequenced. Viruses from serum samples at 7 days PI possess revertant mutations (fig. 2a) suggesting that amino acid residues at these positions undergo a strong selection pressure.

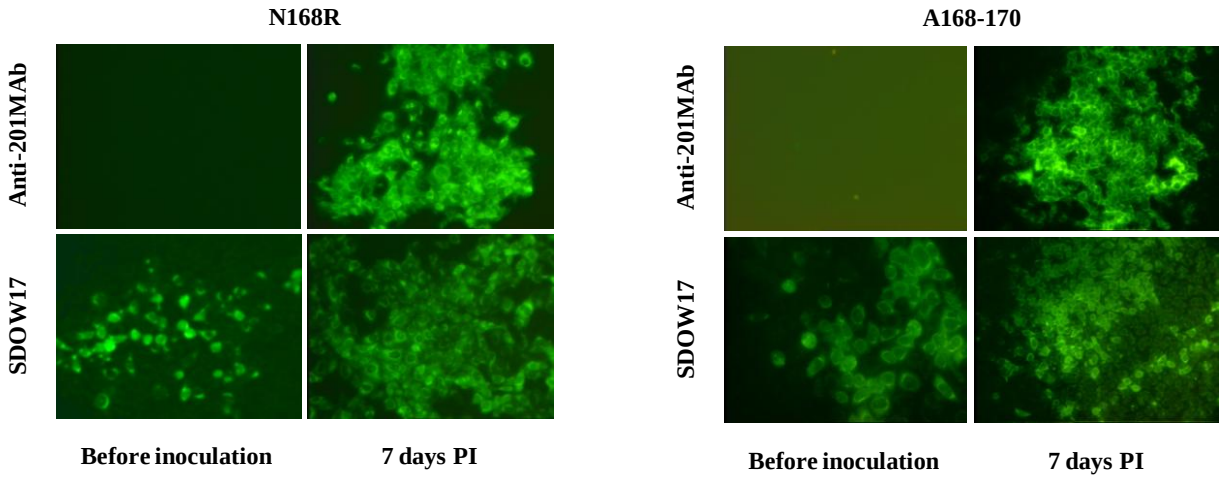

Fig. 1: Indirect immuno – staining using anti-201 MAb. MARC145 cells were infected with indicated viruses or serum samples at 7 day PI. At 48-72h post infection, cells were immune-stained with anti-201 MAb or with SDOW17 (anti-N, indictaing infection by PRRSV). Before inoculation: indicates the fluorescent reactivity phenotype of input virus used to infect the animals. 7 days pi" indicates the fluorescent reactivity phenotype of virus rescued form a viremic sample at 7 days pi.

A

	^{160}KAVKQGVVNLVKYAK^{174}
FL12_ORF6	
N168R_BF_infection	.....R.....
N168R_918-7DPI	.....N.....
N168R_919-7DPI	.....S.....
N168R-922-7DPI	.....N.....
N168R-923-7DPI	.....N.....

B

	^{160}KAVKQGVVNLVKYAK^{174}
FL12wt	
1648	....R.....
17405	R...R.....
17839	R...R.....
MN184	R...R.....
5857	R...R.....
8323	....R.....

Fig. 2: Sequencing analysis of epitope 201 regions. (A) Sequences were obtained from N168R mutant before infection and from serum samples of 4 infected pigs at 7 days PI. (B) Sequences were obtained from 6 type-II PRRSV isolates that do not react with anti-201 MAb.

Objective 3: Field testing of the peptide ELISA specific for the 201 marker epitope

The MAB 201 was used to study the conservation of epitope 201 among a collection of type-II PRRSV field isolates collected from the Mid-west region of the U.S. Of 82 isolates tested, 75 isolates (91.5%) reacted with anti-201 MAb, which indicates that this epitope is highly conserved among type –II PRRSV, although 7 natural isolates (8.5%) tested negative for the marker.
Regarding a diagnostic ELISA test, first version of the test performed as described in material and Methods and used to analyze a sample of 168 sear indicated just a sensitivity of detection of just 31 %. This indicates that much further work for optimization of the test will be necessary. Considering that stability in vivo of a PRRSV marker–negative mutant is priority for this project. we concentrated in that part of the work first.

Discussion

While technically straightforward in the case of some double-stranded DNA viruses, deleting antigen-coding sequences for an entire protein from the genome of a small RNA virus like PRRSV (which encodes only a few proteins, all of them with essential functions) is a difficult task. Thus, the generation of a mutant virus carrying a deletion of an immuno-dominant and conserved small 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.

Our previous experiments demonstrated that deleting sequences of aacids from the epitopic region would be difficult surely due to the vital function that highly conserved sequences may have for the survival of a live strain such our vaccine candidate. We then pursued the generation of mutants with site mutagenesis, without making deletions, that would be negative for the marker activity. We were able to generate 2 different mutant viruses which, without deletions, harbor a disrupted epitope 201. However, both of these mutants were not stable in infected pigs, reverting, after a few days post-inoculation, to wt reactivity, thus not securing a marker-negative serologic response in the inoculated animal (as it would be essential for a DIVA vaccine).

Alternative approaches to ensure a stability of the negative marker in the PRRSV vaccine genome are now being pursued. At the present time we are following a different approach to obtain a stable 201 marker-negative mutant strain of PRRSV. We have conducted analysis of the amino acid sequences of the few PRRSV field isolates (~ 8.5% of the total number of samples anlayzed) that are naturally not recognized by anti-201MAb. Such analysis would permit us to determine which amino acid substitutions would result in disruption of epitope 201antigenic reactivity. After that, we would introduce mutations into FL12 infectious clone which exactly mimic the sequence of the field isolates that are not recognized by anti-201 MAb. We hypothesize that by mimicking the amino acid sequence of a field isolate, we can generate a virus that is stable in infected pigs. All six isolates aligned in such analysis contain a Q164R substitution (see Fig. 2b). Four out of 6 isolates contain an additional K160R substitution. At the time of writing this final report for NEB,#08-248, we are in the process of introducing Q164R mutation into our FL12 infectious clone, under separate, independent funding.