RESEARCH REPORT
SWINE HEALTH

Title: Rational Design of a New Generation of PRRSV Differential (Marker) Vaccines (Renewal, 2007), **NPB 07-232**

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II. Industry Summary

Both industry and scientific experts alike agree in that an effective, broadly protective vaccine against PRRS is a very important tool to help in the control of this disease. Our laboratory pursues the overall objective of developing a new, rationally designed vaccine against PRRSV. For a **"new generation"** vaccine to be successful, it is required that the novel product be **broadly protective**, which means that the vaccine should be able to confer protection against a vast diversity of PRRSV strains that circulate in the field. The project we report herein was initiated in 2005 and continued in 2008, in both cases with Pork Check-off monies. Through this project we sought a novel way for classifying the repertoire of PRRSV strains. The classification of PRRSV strains that we propose is based on the ability of each strain to induce antibodies in a pig or to react with those antibodies in a test tube. By means of a set of selected reference PRRSV strains and specific antisera prepared against these, we have been able to establish a method that compares and classifies the PRRSV strains in their ability to cross neutralize against each other. By "neutralization" it is understood the ability of antibodies to inactivate PRRSV in a test tube. Based on cross neutralization we have been able to define a number of PRRSV strain clusters or groups that we postulate will have direct relationship with the ability of the strains to protect against each other. Under such notion, we can use this information to define the minimal combination of protective specificities **(**or **"valencies"**) that would be required to be contained in a vaccine for this to be **broadly protective**. This NPB project has permitted, by the first time, to describe the variability of PRRSV strains through an objective, biologically meaningful and immunologically measurable parameter. Until now, variability of PRRSV strains had been defined exclusively in terms of genetic sequencing of a small segment of the PRRSV genome (i.e. the GP5 gene). This latter type of measurement does not relate well to actual protection amongst PRRSV strains. Perhaps the most significant output of this NPB-funded project is that it provided the preliminary results that helped to substantiate a larger scale project (of @ 1million dollar) that PRRSV CAP2 recently awarded to a consortium of 4 universities, amongst which we are included. This larger scale project will center on correlating our immunological characterization of the PRRSV strains with the overall variation of their entire genome and their actual cross-protection in vivo.

III Scientific Abstract

A major research goal of our laboratory is the development of a new generation of PRRSV differential marker vaccines. Based on the use reverse genetics technology, we are pursuing the following 3 main objectives: 1) obtain molecularly attenuated vaccine strains, 2) produce a molecular enhancement of the immunogenicity of these novel vaccines and 3) develop a marker differential vaccine system for this new generation of vaccines currently under development. An additional long-term objective began to be addressed by this NPB project **(NPB 07-232)**. Such goal consists of the design of novel vaccines up to standards of satisfactory protective coverage against infection by **homologous or heterologous** PRRSV strains. It is still unclear what defines a heterologous PRRSV strain in terms of protective immunity. Previous work conducted at our laboratory **(NPB 04-174)**, had indicated that there may be serogroups of PRRSV strains defined on the basis of cross-neutralization studies. In this project **(NPB 07-232)**, by use of eight reference strains and their respective mono-specific antisera , we have been able to determine that at least 63 % of all the isolates studied may be typed with at least one of the reference antisera. More importantly, hierarchical clustering analysis of the pattern of cross-reactivity using six of the reference strains allows classifying the entire population of strains studied by us in eight clusters or groups. The patterns of reactivity among these groups vary widely, ranging from one of significant cross neutralization profile (n=1 group), to the minimal or no cross-neutralization profile (n=2). Importantly the prototype strain for the high cross neutralization profile group exhibits a unique pattern of high neutralizing reactivity after inoculation in vivo. Experimental inoculation and molecular studies of this strain indicate that this isolate is a naturally occurring field strain that is stably deglycosylated in one site of both GP3 and GP5 each. Further reverse genetics studies involving this naturally deglycosylated PRRSV strain are ongoing in our laboratory, which may shed light on the role of glycosylation in preventing neutralization as well as the role of GP3 (in addition of GP5) in such function.
This NPB project has permitted, by the first time, to describe the variability of PRRSV strains through an objective, biologically meaningful and immunologically measurable parameter. Until now, variability of PRRSV strains had been defined exclusively in terms of genetic sequencing of a small segment of the PRRSV genome (i.e. the GP5 gene). Perhaps the most significant output of this NPB-funded project is that it provided preliminary results that helped to substantiate a larger scale project (of @ 1million dollar) that PRRSV CAP2 recently awarded to a consortium of 4 universities, amongst which we are included. This larger scale project will center on correlating our immunological characterization of the PRRSV strains with the overall variation of their entire genome and their actual cross-protection in vivo. We anticipate that this research will help to define which sero-groups are important to be represented in the formulation of new vaccines to reach, by single or multivalent combinations, a broad cross-reactive protection.

IV. Introduction

Genetic and antigenic diversity among strains of PRRSV is one of the major obstacles for development of a successful vaccine that could provide a wide spectrum of protection. Sub-typing PRRSV isolates is of cardinal importance for making decisions about which viral strains to use when formulating a vaccine. It has been demonstrated that neutralizing antibodies are important contributors in protective immunity against PRRSV infection. Cross neutralizing activity would therefore be a parameter that allows grouping the constellation of the PRRSV isolates in a manner that would better reflect their antigenic and immunogenic relevance rather than using the mere genetic comparison of selected genes (i.e. ORF5) of PRRSV. Moreover, the value of cross neutralization for establishing subtypes of highly variable RNA viruses has been well proven in case of Foot and Mouth Disease Virus, another highly variable, antigenically multiple RNA virus.

V. Objectives

The objectives originally stated for this project were as follows:

1) To complete the development of a group of high titer, mono-specific convalescent swine sera against selected

isolates spanning the entire genetic/antigenic repertoire of PRRSV US strains

2) To conduct paired cross protection in vivo assays amongst all the sero groups, using representative strains in each case.

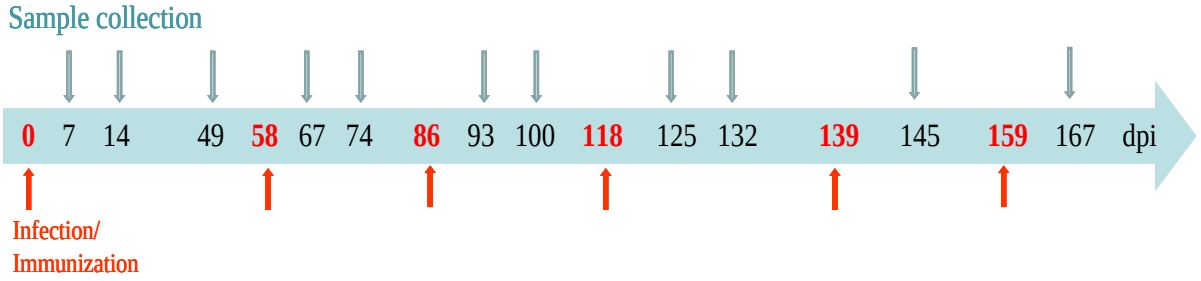
This second objective has been modified in response of the analysis of the results of hierarchical study of seroneutralization profiles and inclusion of this data within the context of a submission to CAP2 request for proposals (see results and discussion)

VI. Materials & Methods

Viruses

Sixty-eight (68) reference and field strains of PRRSV originated from the Mid-West have been used in this study. The PRRSV strains were obtained from Iowa State University Diagnostic Laboratory (contributed by Dr K.J. Yoon) and the University Nebraska – Lincoln Veterinary Diagnostic Center. All of the viruses had been adapted to grow in MARC-145 cells.

Preparation of the antisera:



The diagram above illustrates the hyper-immunization procedure to obtain strain-specific reference antisera in young, immune-competent, PRRSV-free pigs. Complete Freund's adjuvant was used in the first booster immunization (58 dpi) while incomplete Freund's adjuvant was used for the subsequent boosters (shown with red arrows). Serology sample collection times are indicated with grey arrows.

Use of antisera for cross neutralization: Criteria for definition: typable vs. non-typable isolates

Clustering or sero-grouping of PRRSV isolates around one or more reference serums was determined based on their end-point serum-neutralization titer against at least one of the serums. We defined an isolate as being **typable** if the serum-neutralization end-point titer had a vaue of at least 4- fold higher for one serum than against the other reference serums. **Non typable**, instead , were the isolates that exhibited a not such 4-fold difference against any of the reference sera.

Analysis by Reference Antiserums

Eight reference antiserums were developed separately against 8 PRRSV strains which include **Lelystad** (ATCC) : **97-7895** (GenBank accession no. AY545985), **VR2332** (ATCC), **MN184** (source: U of MN, Dr Kay Faaberg) and 4 field isolates **5424-00 1403-02, 3232B-02 and 13867-00** obtained from ISU(Dr. KJ Yoon) The rationale for selecting these particular reference strains was as follows: Four initial isolates, which included the one PRRSV serotype-1 (Euro, Lelystad strain) and 3 isolates of PRRSV serotype 2 (: 97-7895 , VR2332 (ATCC), and MN184)represented the widest range of ORF5 genetic relatedness existing within the spectrum of

US PRRSV type 2 strains, corresponding to approximately a total amplitude of 19 % genetic difference in ORF 5 sequences. Analysis of the entire collection of strains (n=68) by cross neutralization with the antisera prepared against this initial four reference strains was effective at serotyping approximately 50% of the samples. In order to incorporate more strains to our repertoire of typable field isolates, we prepared two additional mono-specific antisera raised, in this case, against 2 selected strains that have been classified by us as untypable in our first round of analyses The isolates used in this case were # 5424-00 and 1403-02. Inclusion of these two antisera to the bank allowed to increase the total number of typable isolates to a total 60.5% of the collection. After this, two additional antisera were prepared against two additional isolates (3232B-02 and 13867-00) that had shown being "non typable" against any of the six previous antisera: isolates. After the inclusion of these two additional antisera the universe of typable isolates increased slightly, to a total of 62.5% of the total strain bank.

VII. Results

Objective 1: Preliminary typing

Sub-typing of PRRSV strains was initially conducted using the results of cross neutralization assays of 68 isolates against 4 or 6 reference antiserums according to the following criteria: one isolate was considered to be "typable" within the group when the end-point titer against the corresponding reference antiserum reached at least 1:64 and was 4 fold higher than the titer against the other antisera. Based on these criteria, we were able to sub-type 49.5 % and 60.5% of the PRRSV isolates studied when either 4 or 6 reference antisera were used. The distribution of PRRSV was as follow: Lelystad: 17.5%; VR2332: 17.5 %; MN184: 5.0%; 97-7895: 3.0%; 1403-02: 16.0% and 5424-00: 1.5%. Therefore 39.5 % of the isolates remained "non typable". Remarkably, inclusion of two additional antisera (3232B-02 and 13867-00, total 8 reference antisera) failed to increase significantly the % of typable isolates. as the total number of nontypable isolates was 37.5%.

Objective 2:

Analysis of the data by hierarchical clustering

During the execution of Objective 1, a hindrance was encountered when attempting to expand the number of typable PRRSV isolates by incorporating new additional reference strains/sera. Such inconvenience is based on the impossibility of predicting the ability of the particular isolate that is selected as reference strain to efficiently induce a robust sero-neutralizing response during the preparation of the reference serum. The SN response produced by the reference strain during the hyperimmunization should be sufficiently high so that a homologous PRRSV-neutralizing titer builds to a level of titer permitting the future use of this serum in typing and differentiating of field strains (i.e. in each case a SN titer of 1:64 or higher should be reached). Failure or sluggishness to build sufficient homologous SN titer in ser, are intrinsic properties to certain strains of PRRSV, as has been previously reported by several laboratories. We postulate that this incapacity is based on our earlier discovery that PRRSV strains may deploy **glycan shielding**, preventing their neutralizing epitopes from inducing neutralizing antibodies (this being a property that varies from strain to strain). In addition, even after finally reaching a suitable titer for the reference antiserum (be this obtained either through repeated lengthy immunizations or by means of enhancing the endpoint titer of the reference antisera through a simple ammonium sulfate precipitation/concentration) one can also find that the number of additional strains that become typable by the addition of the new serum to the reference serum battery could be minimal. As a result, the originally stated goal of typing 100 % of the strains studied incorporating additional new reference strains/antisera may become a lengthy, time consuming and expensive task. We decided then to apply a

hierarchical clustering analysis approach, which would allow us to cluster the strains based on the serum-neutralization results against not just one reference antiserum but based instead on the profile of neutralization reactivity against the entire set of serums composing the reference bank. , When the PRRSV isolates are grouped according to the overall neutralization profiles against the 6 initial reference antiserums, the resulting dendogram is shown in fig.1. Eight clusters were identified, ranging from cluster 8, with significant cross neutralization profile, to cluster 1 & 2, with minimal or no cross-neutralization profiles. **Interestingly, the reference strain 19248-01 (a.k.a. 01, figure 1) which is the prototype strain defining cluster No 8, constitutes, amongst all the strains we studied, the best example of a broadly cross-neutralizing and high inducer of homologous SN titer upon infection. The biology of immune response of this unique strain was studied by animal inoculation and molecular biology, as described in the following paragrap**h.

Further Analysis of Strain 19248-01's Kinetics of Serologic Response

As shown in figure 1, when conducting hierarchical analysis of the serum-neutralizing response profile of strains, we determined that strain (19248-01, or 01) was highly susceptible to all the reference antisera. We sought to find whether this O-1 isolate is able to induce a robust neutralizing antibody. Three weaning pigs were inoculated intramuscularly with the O-1 strain at an inoculation dose of $10^{5.0}$/ pig. Serum samples were collected every week post infection (PI) to evaluate the development of neutralizing antibodies. As shown in fig 2 , the O-1 infected pigs showed robust kinetic of neutralizing antibody development. The neutralizing antibodies were detected as early as 14 days PI. The neutralizing titers sharply increased and peaked at 83 days PI with the titers as high as 1:8192, which is unprecedented for any PRRSV strain reported so far.

We investigated the genetic determinants that influence the robust neutralizing antibody response displayed by this strain. The nucleotide sequence of all structural genes (ORFs 2-7) of O1 strain was determined. Overall, the O-1 shares about 89 to 90% amino acid similarity in structural proteins with VR2332, MN184 and 97-7895 and 60% with Lelystad. The potential N-glycosylation sites in structural proteins of O-1were predicted using the NetNglyc 1.0 server **(http://www.cbs.dtu.dk/services/NetNGlyc/)**. The N-glycosylation sites in GP2 and GP4 are not different from those of other North American PRRSV strains (e.g. VR2332, MN184 and 97-7895). In contrast, GP3 and GP5 possessed altered N-glycosylation patterns. O-1 strain lacked N-glycosylations in GP3 and GP5 which normally appear in VR2332 and 97-7895.

To verify whether GP3 and GP5 were truly hypo-glycosylated, the proteins of O-1 infected cells were immuno-precipitated using PRRSV-specific anti-GP3 and anti-GP5 antibodies and electrophoresized on 12% SDS-PAGE. The GP3 and GP5 of O-1 migrated faster than those of FL12 (infectious clone derived from PRRSV strain 97-7895) demonstrating that they miss one glycosylation site. Based on the preliminary results, we hypothesize that the joint alteration of N-glycosylation pattern in GP3 and GP5 of O-1 may be responsible for its strong capacity to induce neutralizing antibodies s as well as its higher susceptibility to neutralizing antibodies. Currently, and as a logical continuation of these NPB-supported experiments, and under funding from our current USDA-NRICGP grant, we are conducting reverse genetic analysis by means of chimeras between O1 and FL12 strain (Nebraska PRRSV infectious clone)

Whole Genomic Sequencing of Representative Strains of the 8 PRRS Strain Clusters (Figure 1)

Under the premise that the 8 serologically defined clusters (figure1) may constitute a representative sample of antigenic and protective diversity of PRRSV strains, we proposed to use this basic classification, initiated under NPB funding, to further characterize the protective properties of the universe of PRRSV strains, using bio-informatics of the whole genome of PRRSV strains and the crossed-SN phenotypic information as basic classification characters.

To that end we proposed to characterize the complete genome sequence of a sizable number of PRRSV strains, thus inferring the relationship of such genetic spread with the cross-protective spectrum in vivvo. Such proposal was submitted to USDA through the CAP2 competitive program of NRICGP, in response of the recent call for proposals made by Kansas State University. The grant proposed by our 4-site consortium has been successfully awarded (total close to $ 1 million, period of funding 2009/2012) and therefore our first priority became now to obtain the basic full genome information of our most representative 15 strains of PRRSV (2 per each SN cluster, cluster 1 through 7, figure1, plus one for the single defining strain cluster 8, strain 01, figure 1). Thus this new objective takes priority over the cross protection experiments in animals, as originally proposed in this NPB grant. The cross protection analysis will be conducted, instead, under the newly funded CAP2 project in a more substantial, representative way, only after the full genome sequence and analysis of the universe of strains available (target: to obtain at least full genome sequence of 100 PRRSV strains w/i US prototype II of PRRSV)

VIII Discussion

Based on the analysis of typing using a battery of six reference antisera, we were able to sub-type 60.5% of the PRRSV isolates studied. The distribution of PRRSV was as follow: Lelystad: 17.5%; VR2332: 17.5 %; MN184: 5.0%; 97-7895: 3.0%; 1403-02: 16.0% and 5424-00: 1.5%. Therefore 39.5 % of the isolates remained "non typable".

Using hierarchical clustering analysis, we have been able to define 8 clusters of PRRSV isolates representing the complete range of cross neutralization profiles. It is possible that incorporation of additional reference antisera prepared against selected "non-typable" isolates will confirm these clusters, while also being possible that these additional reference antiserums could strongly exhibit cross reactivity with isolates in cluster 1 & 2 (which so far exhibit little or no reactivity with any of the reference antisera).

We are now working at obtaining full genome sequence information of at least one
(but preferably two) representative strains for each cluster . Once we have obtained the full genome sequence of at least 100 diverse PRRSV strains, the next step will be to compare the range of genomic and cross-neutralizing phenotype of the strains representing all the clusters, by conducting bilateral cross – protection studies in vivo using the isolates that represent each of the clusters. This major cross protection study, to be conducted under funding provided by PRRSV CAP2, involves the use of more than 600 pigs.

The overall goal of this project is to define number of valencies or specificities that should be represented in a PRRS vaccine to achieve broad protection.

As a parallel spinoff from this project, the studies of strain 19248-01, which presents an unusual kinetics of serologic response characterized by a hyper-robust and enduring neutralizing response are continued using infectious clone chimeras. The goal in this case is to ascertain the role that deglycosylation of GP5 and GP3 may have in this unusual behavior. Using single gene chimeras we anticipate to characterize the role that GP3 seems to have in PRRSV-neutralization, plus the importance of glycan distribution on the surface of the PRRSV virion envelope in order to facilitate or impair the origination of PRRSV neutralizing antibody response.

VIII. Outputs from this project

1) The following presentation was funded by the NPB 07-232 project :

Sub-typing PRRSV isolates by means of measurement of cross neutralization reactions. 2008. Hiep Vu, Monica Brito, Won-Il Kim, Kyoungjin Yoon , William Laegreid, and Fernando Osorio. Presented at the 89th Conference of Research Workers in Animal Disease, Chicago, Illinois, December 6, 2008. Poster No.88

(It should be mentioned that this presentation received the 2009 first place award granted to the best graduate student poster presentation at CRWAD by the American Association of Veterinary Immunologists)

2) This project provided a significant part of the preliminary results for the following PRRSV CAP2 project that was awarded by Kansas state University subcontract to:

Immunologic Consequences of PRRSV Diversity Laegreid WW (PI) (UIUC) , Osorio, FA(UNL), Goldberg, T(U WI), Christopher-Hennings, J (SDSU) and Nelson, E (SDSU), **$947,885**, funding period: **2009-2012**

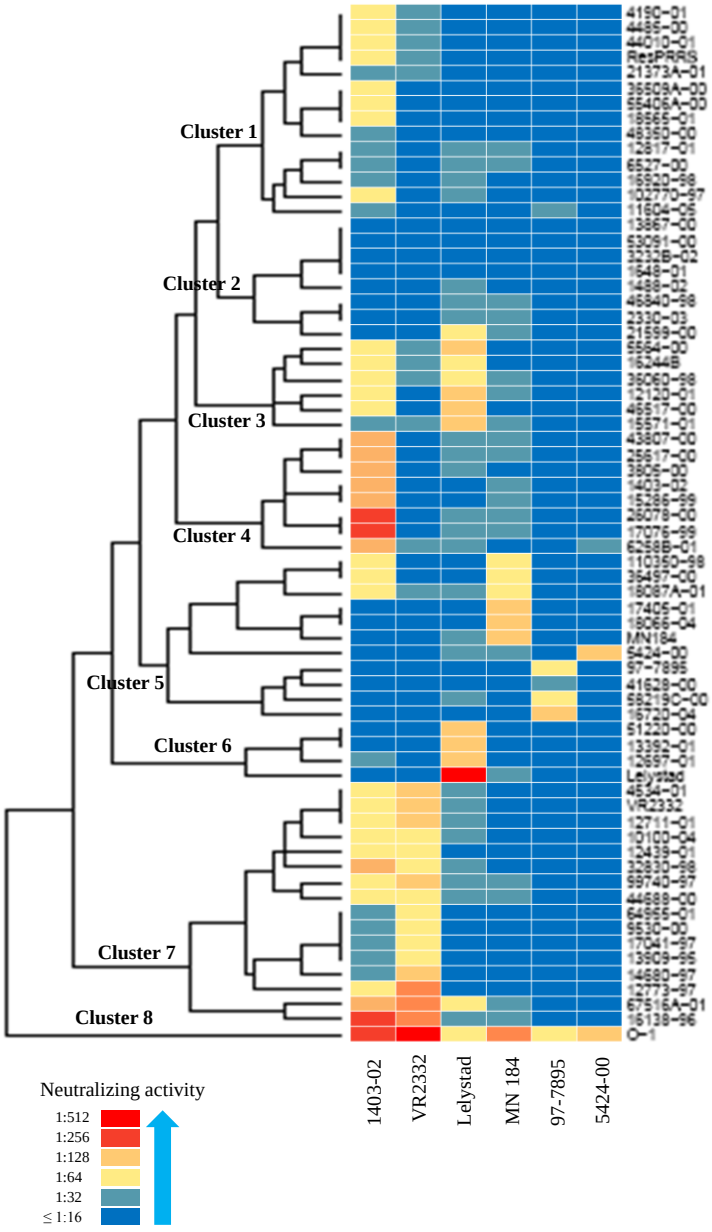


Fig 1: Hierarchical clustering of PRRSV isolates based on cross neutralization profiles . The PRRSV isolates were clustered along the vertical axis. The reference antisera are listed at the bottom.

SN titers against homologous

Days PI	Animal ID		
	2533	6180	7368
14	8	<4	4
21	8	16	16
28	128	128	256
35	512	1024	1024
42	512	1024	2048
49	1024		1024
56	1024	2048	1024
62	1024	2048	4096
76	1024	2048	4096
83	8192	2048	4096
90	8192		4091
104	1024		1024

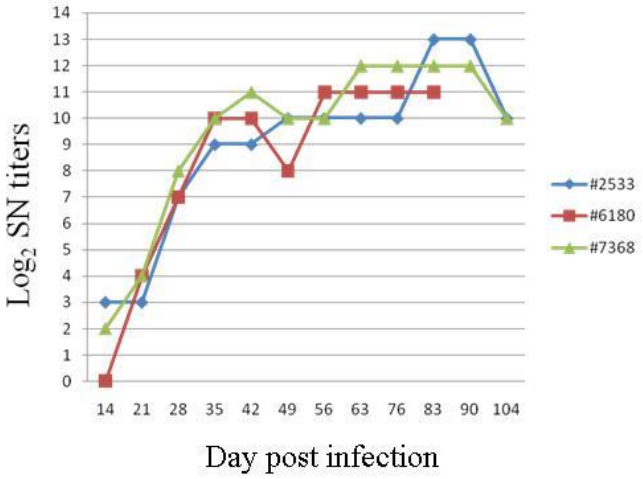


Figure 2: Table and graph above illustrates the unusual kinetics of neutralizing antibody response against the homologous infecting strain (19248-01, a.k.a O-1 strain). Unique characteristics of this neutralizing antibody response (obtained in three different pigs) are:
1) its early character, 2) its intensity, and 3) its persistence throughout time. These infections correspond to a single inoculation of 10^5 $TCID_{50}$ of strain O-1 per pig at day O PI.