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Sub-typing of PRRSV isolates by means of measurement of cross-neutralization reactions

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Title: Sub-typing of PRRSV isolates by means of measurement of cross-neutralization reactions
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Abstract:

The degree of similitude or closeness between two different isolates or strains of PRRSV is very important for deciding which strains should be used for immunization, by either vaccinating with commercial vaccines or attempting to stabilize a herd with planned wt PRRSV infection. As previously known for certain important viral diseases, such as in Foot and Mouth Disease, the reciprocal (cross-) neutralization titers between two strains may be of utmost importance to establish the degree of similarity or difference between these. There is currently a void of methods that would allow distinguishing or grouping strains of PRRSV in a manner that would appropriately reflect immunogenic relatedness and cross protection. Such approach would be a better way of grouping or subtyping isolates than the mere comparison of genetic sequences used nowadays. In this project, the generation of master reference hyperimmune swine antisera against certain selected reference strains of PRRSV allowed us grouping PRRSV strains in some distinct clusters and making inferences about their cross-reactivity and cross-protection by cross-neutralization. These experiments indicate that a high degree of complexity exists in the reciprocal neutralization among strains, suggesting that an increase of the battery of reference serums may be required in order to achieve a complete typing of the universe of PRRSV strains circulating in the U.S. Most importantly, these experiments have provided evidence of the occurrence of unexpected cross- reactivity between otherwise distinct types of PRRSV, thus indicating either the existence of groups of strains of highly atypical (yet undefined) reactivity, or the existence of strains that may emerge from recombination between the two main types of PRRSV.

Introduction:

Protection against PRRSV infection is a matter of primary importance for swine producers. Early studies have indicated that the repertoire of PRRSV isolates in North America is very vast . It was also realized early on that PRRSV immunity is effective mostly against re-infection with homologous strains and, to a lesser extent, against infection with heterologous strains. The great degree of variation exhibited by PRRSV seems to be a characteristic of this agent, and PRRSV is often cited as a typical example of an RNA virus that suffers "antigenic drift ". The antigenic and genetic diversity that results in the antigenic drift observed among the strains of the US type of PRRSV is considered to be one of the major blocks for the development of a successful vaccine that could provide a wide spectrum of protection. The degree of similitude or closeness between two different isolates or strains of PRRSV is of cardinal importance for deciding which strain should be used for immunization, by either vaccinating with commercial vaccines or by attempting "to stabilize" a herd with planned wt PRRSV infection. Currently, the percentage of genetic relatedness between strains is assessed at the level of ORF5, a highly variable gene of PRRSV. Such genetic relatedness is obtained by the determination of the genetic sequence of ORF 5. Genetic relatedness is currently the parameter which is commonly used to establish relative closeness or distance between two given PRRSV isolates. However, the real significance that this percentage of genetic relatedness would have as an indicator of actual antigenic or protective level differences among strains is still unknown. Current ELISA assays can not establish antigenic differences among PRRSV strains, and the existing monoclonal antibody banks are incomplete and therefore unfit for the development of reliable libraries of monoclonals (monoclonals are special antibodies frequently used to type or characterize virus strains) that could be used in the comparison of antigenic reactivity profiles among strains .

We started this project under the assumption that specific antibodies neutralizing PRRSV may provide a significant parameter that would help to establish a clear clustering or sub-grouping of PRRSV strains by cross neutralization. For many years it has been known that other highly variable RNA viruses (i.e. Foot-and-Mouth Disease-FMDV-, a picornavirus) could be sub-typed precisely by means of cross neutralization .Cross neutralization is the measurement of the relative ability of certain immune sera to neutralize homologous and heterologous FMDV strains respectively. In this case "homologous" strain is the same isolate used to prepared the antiserum against, while "heterologous" are those isolates not so related to or frankly distinct from this strain. The seven existing serotypes of FMDV can then be clearly classified and divided, based on cross-neutralization reactions, into several subtypes, each exhibiting varying degrees of cross-protection against the other subtypes within a given serotype. The antigenic relatedness of FMDV strains so assessed has proven vital for identification of antigenic coverage of FMD vaccine strains and for making decisions about incorporation of a given subtype into the formulation of a vaccine. Such an application would also be an essential element in PRRSV vaccination and PRRSV planned exposure plans. For this project we then decided to use mono-specific ("mono-specific" meaning : "prepared against one single PRRSV strain") hyperimune sera raised in swine against selected reference strains within the US type of PRRSV to characterize and compare (through the relative ability of these sera to neutralize the PRRV isolates) the antigenic reactivity profile. Our initial assumption was that such approach would allow the development of a classification of PRRSv isolates in a more biologically/immunologically significant manner than the sequencing methods currently in use.

Objectives:

No.	Objective	Expected Outcomes
1	**To develop a group of high titer, mono-specific convalescent swine sera against selected isolates spanning the entire genetic repertoire of PRRSV US strains**	To obtain reference sera for cross-neutralization studies
2	**To use the reference sera for cross-neutralization studies of a wide range of wt PRRSV strains**	To define PRRSV subtypes, clustering the isolates in base of cross neutralization studies

Materials & Methods:

The following reference PRRSV strains were obtained, propagated and prepared for animal inoculation:

1) PRRSV 2332 (origin: ATCC)
2) PRRSV 97-7895 (Osorio et al. *Virology* 302(1): 9-20,2002)
3) PRRSV MN 184 (kindly provided by Dr. Kay Faaaberg, U. of MN)
4) PRRSV LV(Lelystad) Euro-PRRSV prototype (obtained from NVSL/USDA/APHIS).

These 3 US strains and the one European prototype strain were selected because , upon consultation with different experts, we came to the conclusion that that this group of 4 strains represents the widest possible range of genetic distance, in relation to their GP5 sequence, amongst PRRSV strains. Figure 1 shows the genetic dendogram (a "dendogram" is a chart expressing distance between strains) and Table 1 shows the % of homology amongst these strains.

Pairs of animals were infected by intra-muscular inoculation with each one of these four strains and upon recovery and convalescence were subjected to immunizations (at $\geq$ 60 days post-infection) using a second dose of the respective PRRSV strain delivered in Freund's adjuvant. The serum neutralizing titer developed in the injected animals by each strain was monitored . The treatment was repeated at least once for all the animals and an additional injection was used in selected cases. This methodology was followed until the specific neutralizing titer was high in all cases. The second and third immunizations were interspersed through one month period to allow maturation of the serologic recall response. Once a sizable neutralizing titer was developed in each animal, these were killed and bled out to obtain stocks of each reference antiserum which were fractioned in aliquots, frozen and appropriately stored for this study and for future reference use. The homologous and cross-neutralizing (heterologous) reactivity of these sera is shown in Table 2 .

The reference sera so prepared (objective 1) we used to analyze a sizable large number of field samples as to their 90 % end-point of neutralizing activity against each of the 4 reference antiserums(Objective 2). We were able to develop a collection of 68 field strains thanks primarily to the kind collaboration of Dr. K.J. Yoon 's lab in Iowa State University. His lab transferred to us a large collection of US strains of PRRSV that had been adapted to grow in MARC 145 cells, thus able to be used of the SN assay in such cells. Once we received the strains from Dr Yoon's lab, we titrated and grew all of them to achieve the required infectious titer in each case

in order to use these for the 90 % endpoint SN fluorescent assay, as performed in SDSU lab (Wu et al *Virology*. 2001, 287:183-91)

Results:

The main pattern of cross-neutralization activity among the 4 reference serums and strains is shown in table 2. The homologous end-point titer for each of the 4 antisera consisted, in all cases, of a value several-fold higher against the homologous strain used for hyper-immunization than the value against any of the 3 other heterologus strains. The homologous titers of the reference antisera exceeded those of cross neutralizing heterologous strains within an ample range, varying from 4-fold (i.e. : PRRSV MN 184), to 32-fold (i.e.:PRRSV 97-78795 or Lelystad antisera respectively).
Table 3 shows the end-point titers of a population of 68 PRRSV strains against each of the four reference antisera. This population of strains included both laboratory reference strains as well as field isolates collected throughout the Midwest. To sort these strains according to their SN reactivity against the 4 reference antisera, we defined as prevalent or primary reactivity those end-point titers that were all above of a 1:64 threshold and that presented an end-point titer at least 4-fold higher than any of the remaining antisera. Under those conditions , 36/68. (~ 53 %) isolates could be classified within one of the four groups defined by our selected antisera, as shown in figure 2. On the other hand, 32/68 (~ 47%) would be, under these conditions non-typbale by our system.

Discussion:

From the analysis of table 3 and figure 2, it can be concluded that in the case of 53 % of the isolates studied, the PRRSV strain reacted predominantly with just one of the 4 serums, thus justifying their inclusion in the cluster or group defined by the antiserum. The remaining 47% of the samples did not predominantly react with any serum in particular or did poorly (lat the level $\leq$ 1: 32) with two or more sera. According to the definition that we adopted, these latter samples should then considered as "untypable" by this current system of four reference sera. The grouping of the 53% of samples that fall within the category of "typable" is shown in figure 2. While limited to approximately a little over 50 % of the universe of strains studied, these four clusters shown in figure 2 are useful to ask whether there is any relationship between the sequence of these strains and the reference strain for the cluster , and more importantly , among themselves. Collaborative efforts are currently ongoing between our lab at the University of Nebraska and Dr KJ Yoon's lab at Iowa State University to ascertain the complete structural gene sequence of these isolates which should indicate which gene may be related to the antigenic clustering shown in figure 2.

One intriguing point has been raised by the unexpected reactivity of the anti-Lelystad antiserum with several (at least 9 of them, see table 3) US strains. In these cases the Lelystad strain was the predominant, highest and single reactivity. Initial PCR and sequencing done during the diagnostic isolation of these strains would indicate that these strains responded indeed to the overall genetic features of PRRSV US strains(Dr KJ Yoon , Iowa State University, personal communication). One obvious question that surfaces is whether during the preparation of our Lelystad antiserum there was some possible contamination with one of the other (US) strains. To such concern we respond by remarking that analysis of the cross-reactivity of the Lelystad antiserum against the other three strains used in the study indicates that the titer is very specific and very lowly cross reactive against the 3 US strains, thus arguing against a possible contamination with any of these strains during the manufacturing of the Lelystad serum. It is important to consider that no other PRRSV strain was manipulated in our research lab during the last 5+ years. Therefore the possibility of contamination with any other US strain different from the 3 used in these studies would be out of the question. Complete genome analysis of these strains, currently ongoing in collaboration with Iowa State University scientists (under different funding

source), would help to shed light on the true character of this unexpected reactivity. The possibility of occurrence of a truly inter-typic recombinant can not be ruled out at this time.

The observation that almost half of the field isolates analyzed by us remained as "untypable" (as no predominant reactivity against these four sera could be detected) suggest that we would need to add more reference sera to our reference battery . It seems reasonable that these "untypable" isolates may represent different "clades", as has been described for HIV-1. Alternatively, it is also possible that they correspond to "neutralization resistant" strains, which could be supported by the recently published notion that wt PRRSV is subject to the phenomenon of "glycan shielding" , thus protected, by glycosylation, from or refractory to antibody neutralization (Ansari et al. *J Virol*. 2006 :3994-4004). It will be interesting to sequence these strain in order to determine which gene segregates with a neutralization clade. This type of information is important for the design of targets of new generation vaccines, ascertaining which antigen of PRRSV induces cross neutralization.

Lay Interpretation:

One of the major obstacles for PRRSV control is the significant variability of this virus in the field. Such variability of PRRSV is expressed at the level of the genetic information of the strains (commonly detected in the diagnostic labs by PCR followed by gp5 sequencing) and also, and most importantly, in the antigenic(structural proteins) make-up of the PRRSV strains which determines the strain's ability to induce specific protective immunity in the pig. There is a significant need for typing and classifying PRRSV strains by some means different than the genetic sequencing now in use. The possibility of having a typing system based, rather than in genetic sequencing, in the distinct ability of strains for inducing similar or different immune responses is a very senseful way to classify and group the large universe of PRRSV strains circulating in the field. To our knowledge this project is the first attempt worldwide to apply such a typing system for PRRSV. Using a system of reference antisera that we designed under the assumption that the main PRRSV glycoprotein 5 of PRRSV plays a central role in antigenic diversity and neutralizing activity of PRRSV we were unable to type 50 % of the samples analyzed. While the typing done so far is incomplete, the results obtained, when joined with sequencing studies currently ongoing under different collaborative projects with SDSU and ISU should shed light on the role of specific structural genes of PRRSV in cross-neutralization , typing and probably cross protection. Such information could then be used to increase the number of reference antisera to be incorporated in our library so to maximize the number of "typable" isolates. Likewise, a closer analysis of some strains with anomalous reactivity detected by this research should teach us about the possibility of occurrence of inter-typic recombinants (i.e PRRSV strains originated by crossing of Euro X U.S. types) that may be occurring in the field. This latter information is also crucial for PRRSV control, as there is ample evidence for the simultaneous circulation of both serotypes within the same geographic areas of the US. For further information please contact: Fernando A. Osorio, University of Nebraska-Lincoln, fosorio@unl.edu, phone: 402-472-7809

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Table 1 (percentage of homology) and Figure 1(genetic relationship) between the GP5 gene of the four reference strains used to prepares four mono-specific antisera used in these experiments

ORF 5 (aa)

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	97-7895	200	2	VR-2332	200	91
1	97-7895	200	3	MN184	200	87
1	97-7895	200	4	Lelystad	201	57
2	VR-2332	200	3	MN184	200	84
2	VR-2332	200	4	Lelystad	201	56
3	MN184	200	4	Lelystad	201	55

Figure 1.

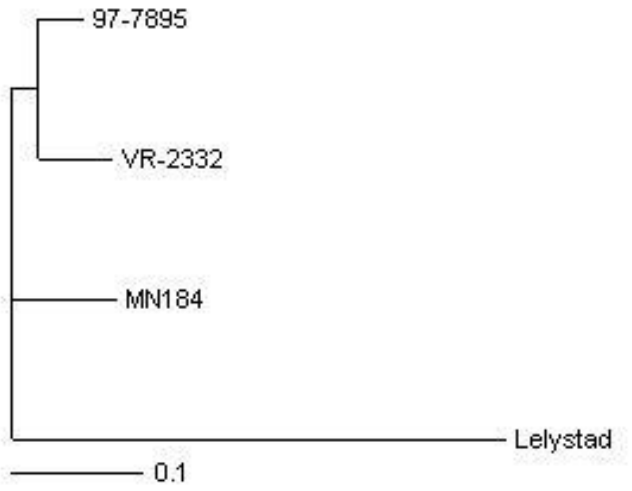

Table 2 Endpoint titers obtained between the four reference antisera prepared and the four reference PRRSV strains

	Virus strain used for the SN assay ? ? ? ?			
Antisera Against ?	**PRRSV 124**	**PRRSV VR2332**	**PRRSV 97-7895**	**Lelystad**
PRRSV 184	**1:128**	1:32	1:32	1:32
PRRSV VR2332	1:13	**1:256**	1:32	1:16
PRRSV 97-7895	1:32	1:32	**1:1,024**	1:32
Lelystad 109 dpi	1:16	1:16	1:8	**1:512**

Table 3 : Results of Cross Neutralization : Numbers correspond to endpoint titers of each of the 4 reference antisera against each of 71 PRRSV strains (including reference and field stains)

Virus Strain ▼	ANTISERUM			
	Lelystad	**97-7895**	**VR2332**	**-MN184**
Lelystad	512	16	16	8
21599-00	128	8	16	4
12120-01	128	4	32	8
12697-01	128	16	32	4
6527-00	512	32	16	8
13392-01	64	4	16	16
46517-00	64	2	8	4
39194-99	32	4	8	4
5564-00	32	2	16	4
55406A-00	8	2	2	2
15386-99	16	4	8	4
5424-00	32	8	16	8
51220-00	64	4	16	8
25617-00	64	2	32	8
1488-02	64	2	32	4
43087-00	32	16	16	4
5966-00	32	4	16	4
6258B-01	16	2	8	4
2330-03	32	8	16	16
97-7895	16	1024	64	32
41628-00	32	128	8	8
16720-04	16	256	8	4
2660-02	16	16	8	4
53091-00	8	16	16	8
13867-00	8	16	8	4
58219C-00	16	64	8	4
48305-00	8	16	8	4
11604-05	16	16	8	4
VR2332	32	32	256	16
4485-00	32	16	128	4
12439-01	64	8	128	4
9530-00	32	16	128	4
4190-01	32	32	256	4
44010-01	16	8	128	4
64955-01	32	8	128	2
4534-01	4	4	128	4
12711-01	32	4	256	8
19248-01	64	32	256	64?
18087A-01	32	8	128	8

Cont. Table 3 Virus Strain ▼	**Lelystad**	**97-7895**	**VR2332**	**-MN184**

1356-00	16	4	128	2
RespPRRS	16	8	128	8
14680-97	16	8	256	4
36497-00	32	2	32	4
17076-99	32	2	32	4
3805-00	16	16	64	8
3233-02	32	32	32	8
36509A-00	32	32	32	8
26078-00	16	8	16	8
12817-01	32	4	32	2
3232B-02	8	8	8	4
21373A-01	16	16	64	4
18565-01	16	8	16	4
1648-01	16	8	16	4
15571-01	32	4	32	8
67516A-01	16	4	32	4
1403-02	16	16	16	4
44688-00	32	2	64	8
5252-02	16	4	32	4
18066-04	16	2	16	8
12773-97	4	4	64	4
17041-97	16	8	64	4
17839-04	16	2	32	16
16138-96	16	4	64	4
13909-05	8	4	64	4
10100-04	16	4	32	2
16244B	32	8	64	4
PRRSV MN 184	32	32	32	128
17405-04	8	4	8	16

Figure 2: Clustering of diverse US isolates around one of the 4 main reference serums used, based on their end-point SN titer against this serum (Based on predominant cross-titer , consisting of the end-point being at least 4-fold higher than against any of the other 3 reference serums)