RESEARCH REPORT
SWINE HEALTH

Title: An Interferon-inducible Porcine Reproductive and Respiratory Syndrome Virus Isolate – **NPB #11-106**

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Industry Summary: The objective of this project was to determine the full length sequences of an interferon-inducible PRRSV strain A2MC2 and determine whether it can induce neutralizing antibodies. Type I interferons (IFNs), such as IFN-α and -β, are critical to antiviral innate immunity and play important roles in the modulation of adaptive immunity. In this study, full length sequence of the A2MC2 genome was determined. Sequence analysis indicated that it is highly homologous to VR-2332, the prototype of North America PRRSV genotype. A2MC2 induction of neutralizing antibodies was compared with the Ingelvac PRRS modified live virus (MLV) vaccine strain and VR-2385 (a moderate virulent strain). Three-week-old pigs were exposed to these PRRSV strains via intranasal or intramuscular routes to also account for a possible effect of inoculation routes. The A2MC2 resulted in earlier onset and significantly higher levels of PRRSV neutralizing antibodies than the MLV. In addition, the A2MC2-induced neutralizing antibodies were capable of neutralizing VR-2385, a heterologous strain. The pigs exposed via intranasal route had higher titers of neutralizing antibodies than those injected via intramuscular route. These results indicate that PRRSV A2MC2 is able to induce higher level neutralizing antibodies, which may be because of the strain property in interferon induction. This information will be helpful in designing an improved vaccine to combat PRRS. For further information, please contact Dr. Zhang at zhangyj@umd.edu.

Keywords: Porcine reproductive and respiratory syndrome virus, PRRSV, neutralizing antibody, IFN-inducing A2MC2, vaccine.

Scientific Abstract:

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) continues to cause substantial economic losses to the global swine industry. PRRSV appears to inhibit synthesis of type I interferons (IFNs), such as IFN-α and -β, which are critical for the innate immunity and play an important role in the modulation of adaptive immunity. An atypical PRRSV strain, A2MC2, is able to induce type I IFNs *in vitro*. In this study, full length sequence of the A2MC2 genome was determined. Sequence analysis indicated that it is highly homologous to VR-2332, the prototype of North America PRRSV genotype. An infectious clone of A2MC2 was constructed and rescued virus was able to induce interferon synthesis in infected cells. A2MC2 induction of neutralizing antibodies *in vivo* was compared with the Ingelvac PRRS modified live virus (MLV) vaccine strain

and VR-2385 (a moderate virulent strain). Three-week-old pigs were exposed to these PRRSV strains via intranasal or intramuscular routes to also account for a possible effect of inoculation routes. The interferon-inducing A2MC2 resulted in earlier onset and significantly higher levels of PRRSV neutralizing antibodies than the MLV in either inoculation routes. In addition, the A2MC2-induced neutralizing antibodies were capable of neutralizing VR-2385, a heterologous strain. The pigs exposed via intranasal route had higher titers of neutralizing antibodies than those injected via intramuscular route. Macroscopic and microscopic lung lesions 14 days post-exposure indicated that A2MC2 had similar virulence *in vivo* as VR-2385. Pulmonary alveolar macrophages (PAMs) collected during the necropsy 14 days post-exposure in the A2MC2 group had higher level expression of IFN-γ than the MLV group. These results indicate that A2MC2 can be further explored for development of an improved vaccine against PRRS.

Introduction:

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease, causing an estimated $664 million loss per year to the swine industry in the United States (15). The causative agent of this disease is PRRSV, an enveloped, single-stranded, and positive-sense RNA virus (5, 24). The genome of PRRSV is a little over 15 kb in length with nine open reading frames (ORFs) (9, 25). Current management strategies and commercial live or autogenously killed vaccines have generally proven inadequate for long-term control of PRRS. Modified live virus vaccines have been used widely to prevent the virus infection since 1990s. However, outbreaks of PRRS resulting from virus strains nearly identical in sequence to the vaccine strains were reported (7, 29, 37). The outbreaks of atypical or acute PRRS in vaccinated pigs have raised serious concern about the efficacy and safety of the current vaccines (21, 22), which demonstrates the need to define PRRSV protective immune response and develop improved vaccines.

PRRSV-infected pigs develop delayed appearance of neutralizing antibodies (19) and weak cell-mediated immune response (45). Neutralizing antibodies against PRRSV correlate with protective immunity (30). Passive transfer of PRRSV-neutralizing antibodies in pregnant sows confers sterilizing immunity against reproductive failure induced by virulent strain challenge. Passive transfer with PRRSV-neutralizing antibodies in young weaned pigs blocks viremia (20).

PRRSV inhibits synthesis of type I interferons (IFNs) in infected pigs (1, 19). IFNs could not be detected in the lung of pigs in which PRRSV actively replicated. PRRSV infection of PAMs and MARC-145 cells *in vitro* leads to very low IFN-α expression (1, 27). Suppression of innate immunity is believed to be an important contributing factor to the PRRSV modulation of host immune responses. Addition of interferons to cultured cells inhibits PRRSV replication (1, 31, 35). Type I IFNs, such as IFN-α and -β, acting in concert with IFN-γ, are critical to innate immunity against viruses and play an important role in the modulation of adaptive immunity (38).

In our earlier study, a novel strain designated as A2MC2 was found to induce type I IFNs in both MARC-145 cells and PAMs (28). A2MC2 induces synthesis of IFNs and expression of IFN-stimulated genes. A2MC2 infection of both types of these cells has minimal effect on the ability of exogenous IFN-α to induce antiviral response. Yet the competence of this IFN-inducing A2MC2 in eliciting neutralizing antibodies in pigs and its virulence *in vivo* are unknown.

The objective of this study is to test the competence of A2MC2 in eliciting neutralizing antibodies in pigs. As A2MC2 induces type I IFNs, the hypothesis was that it would induce an earlier onset and significantly higher titer of neutralizing antibodies than the MLV vaccine strain.

Objectives:

1) To determine the full-length sequence of this novel IFN-inducible PRRSV isolate and conduct sequence analysis.

2) To identify the sequence variation of A2MC2 that lacks an antagonist to IFN production.

3) To determine the immunogenicity of A2MC2 in PRRSV-negative piglets.

Materials & Methods:

a. Cells and viruses

MARC-145 (16) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The PRRSV strain VR-2385 (23), a moderately virulent field strain, a licensed modified live vaccine strain Ingelvac PRRS® MLV, and VR-2332 (5), the parent strain of Ingelvac PRRS® MLV, were used in this study. Virus titers were determined in MARC-145 cells for the median tissue culture infectious dose ($TCID_{50}$) as described previously (47).

b. Sequencing

A2MC2 genomic RNA was isolated from cell culture supernatant with TRIzol LS reagent (Life Technologies). Reverse transcription of the viral RNA was done with Maxima reverse transcriptase (Thermo Fisher Scientific). PCR amplification was done with Phusion high-fidelity DNA polymerase (New England Biolab, Ipswich, MA). 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE of A2MC2 genome were done as previously described (36). Sequencing of the PCR products was performed with ABI Prism 3130 Genetic Analyzer (Life Technologies). Sequence assembly and analysis was done with LaserGene Core Suite (DNASTAR Inc., Madison, WI). The GenBank accession number of cDNA sequence of A2MC2 genome is JQ087873.

c. Animal study to determine neutralizing antibody induction in pigs

The Institutional Animal Care and Use Committees (IACUC) of the University of Maryland and Iowa State University approved this animal study according to relevant guidelines and policies for the care and use of laboratory animals. Three-week-old PRRSV-negative piglets were randomly divided into eight groups and rooms (Table 1). The piglets in groups 1 to 3 were inoculated with 1 ml of PRRSV A2MC2, MLV and VR-2385, respectively, at 10^5 $TCID_{50}$/ml via intranasal inoculation (I.N.); groups 5 to 7 were inoculated with these three strains via intramuscular route (I.M.); while groups 4 and 8 were mock-infected. Three days after the inoculation, two non-treated contact control pigs were commingled with each of the PRRSV-infected groups to evaluate PRRSV shedding and transmission. Phosphate-buffered saline (PBS) pH7.2 was used for mock-infected controls. The pigs were observed daily.

All piglets were weighed three days prior to inoculation and at the time of necropsy 14 or 56 days post-infection (DPI). Blood samples were collected on three days prior to inoculation, and weekly thereafter. Serum was separated and stored at -80°C for subsequent determination of presence and amount of PRRSV viral RNA and neutralizing antibodies.

Pigs were humanely euthanized on 14 or 56 DPI by pentobarbital overdose (FATAL-PLUS, Vortech Pharmaceuticals, LTD. Dearborn, MI). A complete necropsy was performed and visible macroscopic lung lesions were scored and recorded as previously described (13, 14). Lung lavage was collected during necropsy and PAM isolation was conducted as previously described (34). The cells were frozen at -80°C in medium containing dimethyl sulfoxide (DMSO). Lung histopathology was done as described (13, 14). The level of interstitial pneumonia was scored ranging from 0 (absent) to 6 (severe diffuse interstitial pneumonia). Scoring of macroscopic and microscopic lung pathology was done in a treatment status-blinded fashion independently by two veterinary pathologists. If results disagreed, they were combined and the average was used for further analysis.

d. Serum neutralization assay

Serum neutralization assay was performed on MARC-145 cells, as described previously (39, 46) with modifications. Serum samples were tested for virus-neutralizing (VN) antibodies against VR-2332 and VR-2385, respectively. The neutralization assay was done against 100 fluorescence forming units of VR-2332 or VR-2385. The starting dilution of serum samples was 1:8. Indirect immunofluorescence assay with a PRRSV N-specific monoclonal antibody was conducted 48 h after inoculation of the cells as described (46). Compared to serum samples from mock-infected pigs, the reciprocal of the highest serum dilution that reduced 50% PRRSV replication was counted as the VN titer. The VN titer of serum samples that failed to inhibit virus replication at the 1:8 dilution was counted as zero. The serum neutralization assay was repeated three times.

e. RNA isolation, reverse transcription and real-time PCR (RT-qPCR)

Total RNA was isolated from serum and PAM cells with TRIzol Reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions. Reverse transcription of RNA and real-time quantitative PCR were conducted as previously described (33, 34). For detection of PRRS viral RNA in serum samples, known PRRSV cDNA was used as a template to generate a standard curve, against which RNA copies in all samples were calculated. For PAM samples, transcripts of ribosomal protein L32 (RPL32) were also amplified and used to normalize the total amount of input RNA. Relative transcript levels were calculated and shown as folds of change in comparison with mock-treated control as described (33, 34).

f. Statistical analysis

Viral copy numbers (log10 transformed) and VN antibody titers (log2 transformed) of individual pigs were analyzed using a mixed liner model for repeated measures (Proc Mixed, SAS 9.2 for Windows, SAS Institute, Cary, NC, USA). Linear combinations of the least squares means for each variable were used in a priori contrasts in assessing the difference between groups at individual time points. One-way analysis of variance (ANOVA) was also conducted to assess significant difference between two groups in cytokine gene expression and macroscopic and microscopic lung lesion scores. A *P*-value of less than 0.05 was considered significant.

Results:

Objective 1. To determine the full-length sequence of this novel IFN-inducible PRRSV isolate and conduct sequence analysis.

RT-PCR was conducted for the whole A2MC2 RNA genome. Sequencing of the cDNA was done and sequence analysis showed that it closely resembles to Ingelvac® PRRS MLV (GenBank ID: AF066183) and VR-2332 (GenBank ID: U87392), strains in genotype 2 PRRSV, at identity of 99.8%. There are a total of 28 nucleotide (nt) variations from VR-2332, resulting in 14 amino acid changes. The nucleotide variations were scattered from nt 4681 to the end of the genome (Fig. 1). The first 4680 nucleotides are identical to VR-2332. There are a total of 34 nucleotide variations from MLV, resulting in 19 different amino acids. Compared to both VR-2332 and MLV, A2MC2 has 15 unique nucleotides scattered from nt 4681 to the end of the genome (Fig. 1). Ten of the unique changes locate in the sequence from nt 4681 to 10037 of A2MC2 genome. The sequence from nt 11667 to 14420 of A2MC2 is the same as VR-2332 except 4 unique nucleotide variations. The sequence from nt 14421 to the end of the A2MC2 genome is the same as MLV except 1 unique nucleotide variation.

At the amino acid level, the A2MC2 variations from VR-2332 locate in nsp3, nsp7, nsp8, nsp10, nsp11, nsp12, GP3, and M; and the variations from MLV locate in nsp1β, nsp2, nsp8, nsp10, nsp11, nsp12, GP2, GP3, GP5, and M. A2MC2 has 6 unique amino acid changes: from threonine in VR-2332 and MLV to serine in A2MC2 in nsp8, serine to alanine and proline to leucine in nsp10, serine to glycine in nsp12, methionine to valine, and isoleucine to valine in GP3 (Table 1). Nsp10 is an RNA helicase to unwind dsRNA (4), while functions of nsp8 and nsp12 are unknown. GP3 is a glycoprotein found in PRRSV virions as a minor structural component (11). The closeness in genome sequence of A2MC2 to both VR-2332 and MLV indicated that A2MC2 was possibly a chimera derived from these two strains.

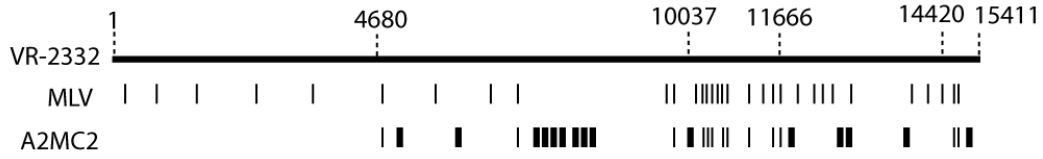

Fig. 1. Illustration of sequence variation of A2MC2 in comparison with VR-2332 and MLV. The top line indicates the genomic sequence of VR-2332 and the numbers above the line indicate nucleotide positions in the genome. The nucleotide variations of MLV in comparison with VR-2332 are indicated by narrow vertical bars. The nucleotide variations of A2MC2 in comparison with VR-2332 are indicated by both narrow and wide vertical bars, among which the narrow vertical bars indicates they are the same as MLV and the wide bars indicates they are unique for A2MC2.

table 1. Nucleotide variations in A2MC2 sequence leading to unique amino acid changes compared with both MLV and VR-2332[a]

Position[b]	Nucleotide[c]			Amino acid[d]			Protein[e]
	A2MC2	MLV	VR-2332	A2MC2	MLV	VR-2332	
7621	T	A	A	S	T	T	nsp8
9627	G	T	T	A	S	S	nsp10
9655	T	C	C	L	P	P	nsp10
12012	G	A	A	G	S	S	nsp12
12972	G	A	A	V	M	M	GP3
12975	G	A	A	V	I	I	GP3

a. GenBank accession numbers: VR-2332 (GenBank ID: U87392), MLV (GenBank ID: AF066183), and A2MC2 (GenBank ID: JQ087873).
b. Nucleotide positions are indicated on left column based on VR-2332 sequence.
c. Nucleotides at the indicated positions are listed.
d. Amino acids derived from the codon of indicated nucleotides are listed.
e. Proteins corresponding to the amino acids derived from the codon of indicated nucleotide positions are listed on the right column.

Objective 2. To identify the sequence variation of A2MC2 that lacks an antagonist to IFN production.

The original assumption was the sequence variations in A2MC2 gene(s) that cause it to lose the inhibition of type I IFN production would be identified. Special attention was planned on the sequence encoding the nonstructural protein NSP1, NSP2, NSP7 and NSP11 as they have been reported to block IFN induction (6, 18). But after the full-length sequence of A2MC2 was available after Objective 1 above was done, we found that the four nsps of A2MC2 are the same as VR-2332. The original experimental design to test these four nsps between the two strains became non-applicable. Thus we modified the experimental plan to construct an infectious clone of A2MC2 for identification of the sequence variations in A2MC2 that lacks an antagonist to IFN induction.

A. Construction of an infectious cDNA clone of A2MC2. The strategy to construct an infectious cDNA clone of A2MC2 is illustrated (Fig. 2). RNA was extracted from A2MC2 virions and used in reverse transcription to synthesize cDNA. The full length cDNA of A2MC2 was cloned into pCAGEN vector as previously described (42). DNA sequencing confirmed the sequence of A2MC2 cloned into the plasmid. Transfection of MARC-145 cells led to recovery of virus from the pA2MC2 plasmid. MARC-145 cells infected with pA2MC2 virus had CPE development (Fig. 3A). PRRSV N protein in the infected cells was demonstrated with PRRSV IFA (Fig. 3B).

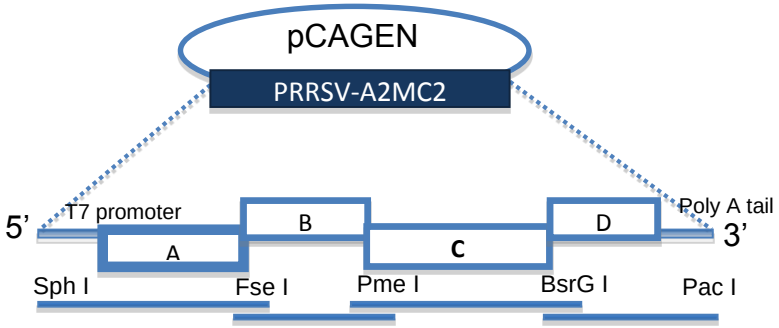

Fig. 2. Strategy for the construction of a full-length infectious cDNA clone of A2MC2. Four fragments (A, B, C, and D) amplified from cDNA of total RNAs extracted from the A2MC2 virus were assembled into pCAGEN vector including T7 promoter and poly (A) tail. Unique restriction enzyme sites used for the assembly of the full-length clone are indicated above

B. Rescued pA2MC2 induces interferons in cultured cells.

The interferon bioassay showed that the supernatant samples from pA2MC2-infected cells inhibited NDV-GFP replication when dilutions up to 1:8 of the supernatant were used to treat Vero cells (Fig. 4). The rescued virus appeared similar to its parent strain in terms of induction of type I interferons and of growth properties in cultured MARC-145 cells. However, the success rate of rescuing virus after transfection was low due to its sensitivity to IFNs, which the virus induces. Further work is needed to make a robust infectious cDNA clone.

Objective 3. *To determine the immunogenicity of A2MC2 in PRRSV-negative piglets.*

Three-week-old PRRSV-negative piglets were randomly divided into nine groups (Table 2). The pigs were exposed to PRRSV via intramuscular (I.M.) or intranasal (I.N.) routes. The two routes of exposure were selected to mimic natural infection by respiratory transmission or vaccine delivery via I.M. injection. PRRSV A2MC2, VR-2385, Ingelvac PRRS MLV and pA2MC2, the rescued virus from the A2MC2 cDNA infectious clone, were used in the study at 10^5 $TCID_{50}$ per pig. Two contact control piglets were placed in each of the first seven groups at three days post inoculation (dpi) to assess PRRSV shedding and transmission. Phosphate-buffered saline (PBS) pH7.2 was used for mock-infected controls. Necropsies were conducted at DPI 14 for four pigs in group 2, 4, 6, 7 and 9 and at DPI 56 for the rest of the pigs.

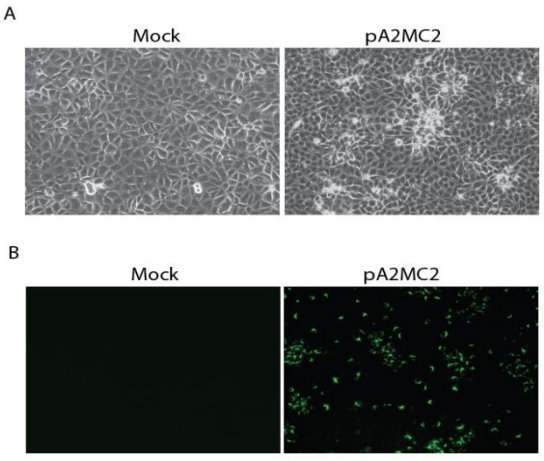

Fig. 3. Characterization of rescued virus from pCAGEN-A2MC2. A. MARC-145 cells were infected with supernatant from rescued pA2MC2 for 48 h, and CPE was observed using phase contrast microscopy. Mock-infected cells were included as controls. B. Immunofluorescence assay (IFA) to detect PRRSV-positive MARC-145 cells infected with rescued pA2MC2 virus. A PRRSV N-specific monoclonal antibody was used in the assay.

Blood samples were collected once a week for the detection of neutralizing antibody and PRRSV RNA. The

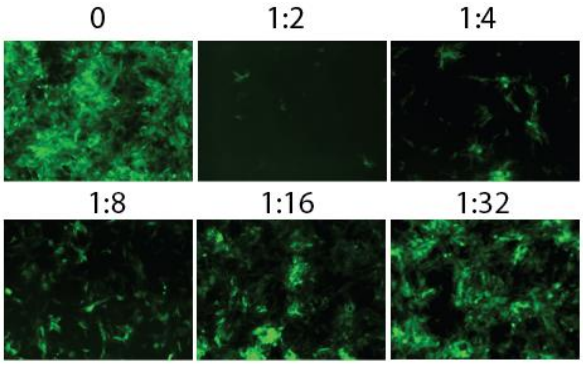

Fig. 4. Rescued pA2MC2 virus induces synthesis of IFNs in MARC-145 cells. Cell culture supernatants from MARC145 cells infected with rescued pA2MC2 virus were collected 48 hpi. Vero cells were treated with a dilution series of supernatants for 12 h, and then infected with NDV-GFP. Fluorescence microscopy was conducted at 24 h after NDV-GFP inoculation. The reduction of GFP-positive cells indicates inhibition of NDV-GFP replication. Note that treatment of the cells with the dilution 1:8 still led to inhibition of NDV-GFP replication.

piglets were weighed before the start of the experiment and immediately before necropsy. To evaluate lung pathology, four piglets in group 2, 4, 6, 7 and 9 were subjected to necropsies on DPI 14. All other pigs were subjected for necropsy at the end of the experiment. Lung samples and other tissues were collected during necropsy for histological examination (13, 14), evaluations of gross and microscopic lung lesions were done in a treatment status-blinded fashion to eliminate subjective bias.

Table 2. Experimental design for the pig study

Event	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Total
PRRSV	A2MC2	A2MC2	VR-2385	VR-2385	MLV	MLV	pA2MC2	PBS	PBS	
Routes	I.M.	I.N.	I.M.	I.N.	I.M.	I.N.	I.N.	I.M.	I.N.	
Number of Piglets	4	8	4	8	4	8	8	4	8	56
Contact control	2	2	2	2	2	2	2	0	0	14

A. Neutralizing antibody titration. Virus neutralization assay was performed on MARC-145 cells, as described previously (2). Serum samples were tested for neutralizing antibodies against VR-2332. A2MC2 infection induced earlier onset and higher titer of virus neutralizing (VN) antibody than MLV and VR-2385 (Fig. 5). The VN antibody in A2MC2-infected pigs was detectable on DPI 28 and increased until DPI 56, the animal study was terminated. Among the pigs exposed via the I.N. route, A2MC2 induced the highest levels of VN antibodies (Fig. 5A). The average VN titers in the A2MC2 group were 2-6 folds higher than those in the MLV group. Among the A2MC2-infected pigs via the I.N. route, two pigs had detectable VN antibodies by DPI 28 and all four pigs had VN antibodies by DPI 56. In the MLV group of the I.N. route, one pig had VN antibody by DPI 35 and all four were positive by DPI 56. Only one VR-2385-infected pig had VN antibody by DPI 56. In the pA2MC2-infected pigs, two developed VN antibodies by DPI 42 and all four by DPI 49.

Among the pigs inoculated via the I.M. route, A2MC2 induced the highest level of VN antibodies (Fig. 5B). Four A2MC2-infected pigs developed neutralizing antibodies at titers of 128, 8, 16 and 64 by DPI 56, while only one out of four MLV-infected pigs had a VN titer of 32 by DPI 56. Among the pigs

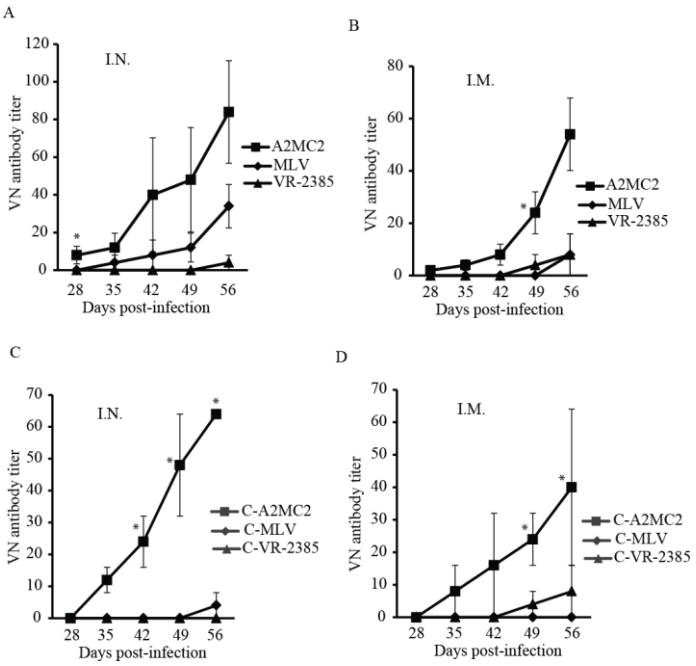

Fig. 5. Evaluation of virus-neutralizing (VN) antibodies against VR-2332. **A**. VN antibody titers in serum samples from pigs exposed via the I.N. route. Average VN titers are shown. Error bars indicate standard errors of mean. The starting dilution of the serum samples was 1:8. The VN titer of those serum samples at 1:8 dilution that failed to reduce 50% virus replication was counted as zero. Statistical analysis shows that overall VN antibody titers in A2MC2 group are significantly higher than MLV group ($P < 0.05$). "*" denotes significant difference ($P < 0.05$) in VN antibody titers between A2MC2 and MLV groups in that time point. **B**. VN antibody titers in pigs exposed via the I.M. route. **C**. VN antibody titers in contact control pigs in groups exposed via the I.N. route. Pigs in A2MC2 group had significantly higher VN antibodies than the MLV group ($P < 0.01$). **D**. VN antibody titers in contact control pigs in groups exposed via the I.M. route. Significant difference ($P < 0.05$) in mean VN antibody titers between A2MC2 and MLV groups at different time points are

infected with VR-2385, only one pig had neutralizing antibody by DPI 56.

For A2MC2 and MLV, the I.N. delivery route elicited higher levels of VN antibodies than the I.M. route; but the difference between the inoculation routes is only statistically significant for the MLV ($P < 0.05$), while the inoculation routes had minimal effect for VR-2385 infection. Trends were similar in contact pigs (Fig. 5C&D). All contact pigs in A2MC2 groups (I.N. and I.M.) had detectable VN antibodies by DPI35, which persisted through DPI56. Only one pig in the MLV contact control groups developed neutralizing antibodies detectable by DPI56.

B. A2MC2 elicits VN antibodies against a heterologous strain

The DPI49 and DPI56 serum samples were also tested in a serum neutralization assay against VR-2385, a heterologous strain for A2MC2 and MLV (Fig. 6A). Three of four I.N. A2MC2 pigs at DPI49 and all four at DPI56 had VN antibodies against VR-2385, while none at DPI49 and one of four MLV-infected pigs at DPI56 had VN antibodies. All VR-2385-infected pigs in the I.N. group had detectable VN antibodies in this assay at both DPI49 and DPI56.

The average titers for serum samples from pigs exposed via the I.M. route in the VR-2385, A2MC2 and MLV groups were 80, 24 and 0, respectively, for DPI49 and 64, 32 and 0, respectively for DPI56 (Fig. 6B). There was no statistical difference between the A2MC2 and MLV groups. Similar trends were observed in contact control pigs (Fig. 6 C&D). These results indicate that A2MC2 elicited higher neutralizing antibodies against heterologous VR-2385 compared to the MLV.

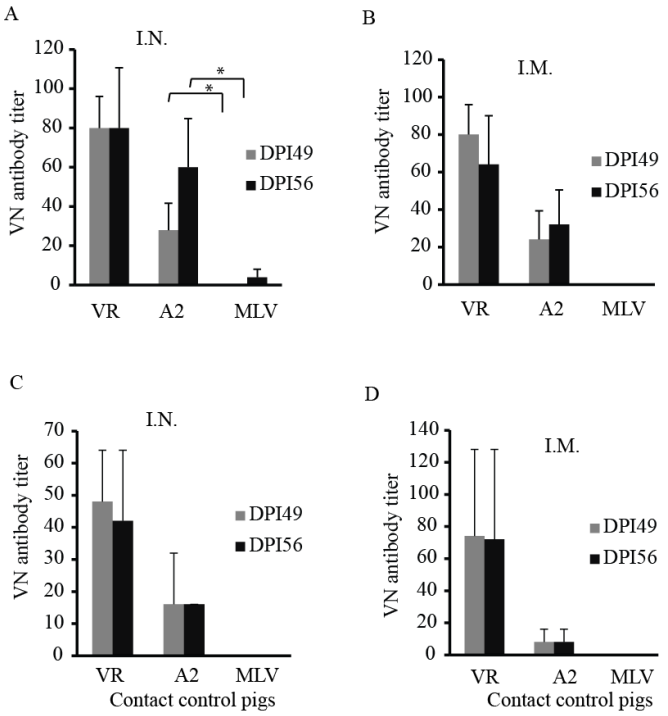

Fig. 6. Evaluation of virus neutralizing antibodies against VR-2385. **A**. VN antibody titers in pigs exposed via the I.N. route. Average group titers are shown. Serum samples collected at DPI 49 and 56 were used in this assay. Error bars indicate standard error of mean. The starting dilution of the serum samples was 1:8. The titer of those serum samples at 1:8 dilution that failed to reduce 50% virus replication was expressed as zero. VR: VR-2385; A2: A2MC2. Statistical analysis shows that overall VN antibody titers in A2MC2 group are significantly higher than MLV group ($P < 0.05$). "*" denotes significant difference ($P < 0.05$) in mean VN antibody titers between A2MC2 and MLV groups. **B**. VN antibody titers in pigs exposed via the I.M. route. **C**. VN antibody titers in contact control pigs in groups exposed via the I.N. route. **D**. VN antibody titers in contact control pigs in groups exposed via the I.M. route.

C. Average daily weight gain. The pigs were weighed on arrival and at necropsy. Average daily weight gain was calculated by dividing total weight gain with number of days for each pig and then for each group. There was no significant difference across the groups (data not shown). The A2MC2 or VR-2385-infected pigs had a similar average weight gain compared to the MLV-infected pigs.

D. Lung lesions.

Among four pigs necropsied at DPI14 for each group, three A2MC2-infected pigs, one MLV infected pigs, and three VR-2385 infected pigs had visible macroscopic lung changes. Overall, the A2MC2-infected pigs showed higher average gross lung lesion scores than the other groups, but the difference was not statistically significant (Fig. 7A). As expected, PRRSV-induced lesions were essentially resolved by DPI56 with only minimal lesions remaining in a few pigs (data not shown).

Microscopically, the interstitial pneumonia scores for the A2MC2-infected pigs at DPI14 were similar to the VR-2385-infected pigs (Fig. 7B). Both the A2MC2 and VR-2385 groups had significantly higher scores than the MLV-infected pigs. The MLV group had no significant difference from the PBS control group. At DPI56, essentially no interstitial pneumonia lesions were observed (data not shown). These results suggest that under the study conditions, A2MC2 infection lead to pneumonia lesions similar to the well characterized VR-2385.

E. Detection of PRRSV replication.

Weekly collected serum samples were used for the detection of PRRSV RNA by RT-qPCR. Pigs infected with A2MC2 and VR-2385 had the highest viral loads in serum by DPI7, but only statistically higher than the MLV on DPI14 in I.N. route and on DPI7 in I.M. route inoculation (Fig. 8A&B). The viral RNA copy numbers decreased after DPI7 through DPI42. Infection with the MLV strain resulted in relatively lower viral RNA loads. The difference in viral RNA copies between I.M. and I.N. routes was minimal.

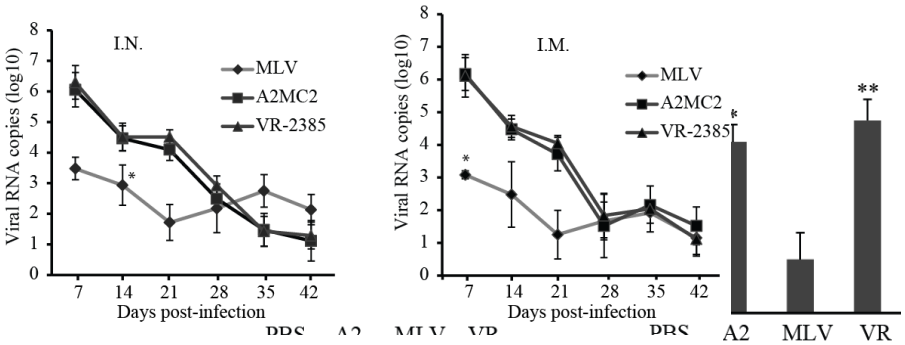

Fig. 8. PRRSV RNA copies in serum samples of different days post-infection detected by RT-qPCR. The pigs were exposed to PRRSV by the IN (A) or IM (B) routes. A standard curve was used for calculation of number of viral RNA copies, which were converted to log10/ml. Average copies of viral RNA in each group are shown. Error bars represent standard errors of mean. "*" denotes significant difference ($P < 0.05$) in VN antibody titers between A2MC2 and MLV groups at that time point.

V A2MC2, MLV, from each group were included as scores. Error bars four pigs in each microscopic lung group of mock- indicate $P < 0.05$ and $P < 0.01$, respectively.

Discussion:

Analysis of cDNA sequence of A2MC2 genome shows that it is highly homologous to both VR-2332 and MLV. This result indicates that A2MC2 might be a chimera of VR-2332 and MLV strains. Based on the sequence analysis, we speculate that the first 4.6 kb and fragment from nt 11966 to 14420 are from VR-2332; the fragments of nt 10697 to 11666 and nt 14421 to the end of the genome are possibly from MLV; and the fragment of nt 4681 to 10037 is from any one of them but with mutations as 10 unique changes are located in this fragment. Compared to both VR-2332 and MLV, A2MC2 has 6 unique amino acids distributed in nsp8, nsp10, and nsp12, and GP3. Based on the sequence comparison, future experiments on mutated viruses using reverse genetic technology will be conducted to pinpoint the A2MC2 sequences that are involved in the loss of inhibition of IFN production.

Neutralizing antibodies against PRRSV correlate with protective immunity (30). A safe vaccine that elicits a robust neutralizing antibody response would likely be widely accepted by the industry. The present *in vivo* study revealed that the IFN-inducing strain A2MC2 elicited an earlier onset and higher titer of VN antibodies in young pigs than MLV. The A2MC2-elicited antibodies were also able to neutralize a heterologous strain.

Growing pigs were used in this study to evaluate the competency of the IFN-inducing A2MC2 to elicit VN antibodies. A2MC2-infected pigs had detectable neutralizing antibodies at DPI28 which continuously increased until termination of the study at DPI56. The MLV strain evoked much lower levels of neutralizing antibodies, which is consistent with previous reports showing its induction of weak immune response (3, 10). Interestingly, the pigs exposed to A2MC2 and MLV via the I.N. route had higher titers of neutralizing antibodies than the pigs infected via the I.M. route. A possible explanation for this finding is that the I.N. route delivery is similar to the natural route of PRRSV exposure.

The serum neutralization assay using VR-2385 showed that A2MC2-infected pigs developed VN antibodies against the heterologous strain. None of the MLV serum samples with the exception of one pig had neutralizing antibodies against VR-2385. These pigs also had low VN antibody titers against homologous strain VR-2332 indicating an overall lower humoral immune response.

While VR-2385 elicited robust neutralizing antibodies against itself, VR-2385-infected pigs developed none or only low titers of VN antibodies against VR-2332. One possible reason for this finding is that the two strains have different VN epitopes. This speculation seems reasonable because sequence analysis showed that ORFs 2 to 5 of the two strains had 94.5% and 94.3% identity at nucleotide and amino acid level, respectively. ORFs 2 to 5 encode GP2, GP3, GP4, and GP5, respectively, which have been reported to be targets of virus-neutralizing antibodies (12, 26, 40, 43, 46). However, sequence homology or lack of thereof cannot explain why A2MC2-infected pigs had cross-neutralizing antibody against VR-2385, whereas VR-2385-infected pigs had minimal VN antibodies against VR-2332, a high homologous strain of A2MC2 (44). Thus, it seems a reasonable explanation that A2MC2 induction of interferons correlates with its elicitation of cross-neutralizing antibodies. Overall, A2MC2 elicited an earlier onset and higher levels of neutralizing antibodies. If challenged, the A2MC2-infected pigs should have been better protected. Further animal studies with A2MC2 are needed to confirm this speculation.

In conclusion, the A2MC2 induced higher level neutralizing antibodies against homologous and heterologous PRRSV strains compared to the MLV strain. In growing pigs, A2MC2 showed moderate virulence similar to VR-2385. Further exploration of A2MC2 to prepare it to be a potential candidate for an improved PRRS vaccine is warranted.

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