RESEARCH REPORT
SWINE HEALTH

Title: Characterization of high passages of an interferon-inducing PRRSV strain**, NPB #15-160**

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Industry Summary: As stated in the contract, we require an industry summary of the project, suitable for immediate public release by the Board. The purpose of the industry summary is to provide producers with a quick reference to research results supported by Checkoff dollars. The content should include the following: an explanation of the objectives, descriptive narrative of how research was conducted, a discussion of the research findings sufficient to give a thorough understanding of the results, and explain what these findings mean to the industry. This summary is to be written for non-technical audiences. Please include your contact information.

The prevalence of PRRSV infection in swine herds is high. Current strategies to control the spread and impact of PRRSV infection once it enters a herd have largely been proven inadequate. Development of new vaccines or improvement of the current vaccines is needed. The typical features of the immune responses in PRRSV-infected pigs are delayed inception and low level of neutralizing antibodies as well as weak cell-mediated immunity. One of the possible reasons is that PRRSV interferes with the innate immunity, including downregulation of type I interferons (IFNs) in infected pigs. Type I IFNs are critical to the innate immunity against virus infections and play important roles in activation of the adaptive immunity. It is fortunate that we discovered an IFN-inducible PRRSV strain, A2MC2, which provides a good opportunity to develop an improved vaccine against PRRS. The virus was serially passaged in cultured cells to passage 90 (A2MC2-P90) in laboratory. The high passage virus is still able to induce IFNs. The objectives for this project are to assess the virulence and efficiency of A2MC2-P90 in elicitation of the host immune response and to construct A2MC2-P90 infectious clone. Nucleic acid sequencing of the A2MC2-P90 genome was conducted. Sequence analysis showed that the A2MC2-P90 has genomic nucleic acid identity of 99.8% to the wild type but has a deletion of 543 nucleotides. The cDNA of the full-length genome of A2MC2-P90 was amplified and assembled for construction of an infectious clone. The establishment of this clone will be useful for further studying the biology of this virus and development of an improved vaccine against PRRS. The A2MC2-P90 virus

was tested in young pigs along with the wild type A2MC2 and Ingelvac PRRS® MLV strain. Inoculation of three-week-old piglets showed that A2MC2-P90 is avirulent and elicits the host immune response. Compared with the Ingelvac PRRS® MLV strain, A2MC2-P90 elicits higher virus neutralizing antibodies. The avirulent IFN-inducing A2MC2-P90 should be useful for development of an improved PRRSV vaccine. Application of such a vaccine will yield significant economic benefits to the swine industry by preventing PRRS. For more information, contact Dr. Zhang at the University of Maryland: zhangyj@umd.edu.

Keywords:
PRRSV, interferon, vaccine, infectious clone, virus-neutralizing antibody, host immune response.

Scientific Abstract:

Porcine reproductive and respiratory syndrome virus (PRRSV) is known to antagonize the production of type I interferons (IFNs). Type I IFNs are critical to the innate immunity against virus infections and play important roles in activation of the adaptive immunity. We recently discovered the atypical strain A2MC2 that is capable of inducing IFN synthesis in cultured cells. The A2MC2 was serially passaged 90 times (A2MC2-P90) for attenuation of its moderate virulence. A2MC2-P90 retains the feature of interferon induction in MARC-145 cells. The objectives of this study were to characterize A2MC2-P90, construct an infectious cDNA clone of this high passage virus, and assess its virulence and immunogenicity in pigs. The A2MC2-P90 replicates faster with a higher virus yield than the wild type A2MC2 virus. Infection of primary pulmonary alveolar macrophages (PAMs) also induces interferons. Sequence analysis showed that the A2MC2-P90 has genomic nucleic acid identity of 99.8% to the wild type but has a deletion of 543 nucleotides in nsp2. The deletion occurred in passage 60. The A2MC2-P90 genome has a total of 35 nucleotide variations from the wild type, leading to 26 amino acid differences. For construction of a cDNA infectious clone of A2MC2-P90, four fragments spanning the full length cDNA of A2MC2-P90 genome were amplified and assembled into a target vector. A DNA-launched version of the infectious clone was generated by adding ribozymes to both ends of the cDNA. Virus rescue was conducted from the infectious clone for the recovered virus, rA2MC2-P90. To test virulence and immunogenicity of A2MC2-P90, three-week-old piglets were inoculated along with the wild type A2MC2 and Ingelvac PRRS® MLV strain. Results showed that A2MC2-P90 is avirulent and elicits immune response. Compared with Ingelvac PRRS® MLV strain, A2MC2-P90 elicits higher virus neutralizing antibodies. The attenuated IFN-inducing A2MC2-P90 should be useful for development of an improved PRRSV vaccine.

Introduction: An overview of the researchable question and its importance to producers.

PRRS causes an estimated $664 million in losses per year to the swine-producing industry in the USA alone (1), an increase of approximately $104 million from the $560 million annual cost estimated in 2005 (2). The causative agent of this disease is PRRSV, an enveloped, single-stranded, and positive-sense RNA virus (3, 4). The genome of PRRSV is a little over 15 kb in length with ten open reading frames (ORFs) (5-7). Current management strategies and commercial live or autogenously killed vaccines have generally been proven inadequate for long-term control of PRRS. Modified live virus (MLV) vaccines have been used widely to prevent the virus infection since mid-1990s. However, outbreaks of PRRS resulting from virus strains nearly identical in sequence to the vaccine strains were reported (8, 9). The outbreaks of atypical or acute PRRS in vaccinated pigs have raised serious concern

about the efficacy and safety of the current MLV vaccines (10, 11), which demonstrates the need to define PRRSV protective immune response and develop improved vaccines.

The typical features of immune responses in PRRSV-infected pigs are delayed onset and low level of neutralizing antibodies (12) and weak cell-mediated immunity (13). PRRSV inhibits synthesis of type I interferons (IFNs) in infected pigs (14, 15). 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 (15, 16). Suppression of innate immunity can be an important contributing factor to the PRRSV modulation of host immune responses.

Many PRRSV strains suppress IFN expression in MARC-145 cells and PRRSV non-structural proteins (nsp) nsp1, nsp2, nsp4, and nsp11, and the nucleocapsid protein inhibit IFN induction when over-expressed (17-25). PRRSV can also inhibit IFN downstream signaling and expression of IFN-stimulated genes (ISGs) in both MARC-145 and PAM cells (26-28).

A novel isolate, A2MC2, induces IFNs in both MARC-145 and PAM cells and virus replication is needed for the IFN induction (29). Type 1 IFNs are found in culture supernatant of A2MC2-infected cells. A2MC2 infection of MARC-145 or PAM cells has no detectable inhibitory effect on the ability of external IFN-alpha to induce an antiviral response. Sequence analysis indicates that A2MC2 is closely related to VR-2332 and the MLV vaccine strain with an identity of 99.8% at the nucleotide level. The A2MC2 induction of IFN is dose-dependent in MARC-145 cells. The virus replication is robust when the inoculum is at a MOI (multiplicity of infection) as low as 0.01 (29).

Virus-neutralizing antibodies against PRRSV correlate with protective immunity. Passive transfer of PRRSV-neutralizing antibodies confers sterilizing immunity in pregnant sows against reproductive failure induced by virulent strain challenge (30) and blocks viremia in young weaned pigs (31).

In developing vaccines against PRRSV, attenuated live vaccines, killed vaccines, subunit vaccines and other methods have been tried. From the perspective of swine production and cost of vaccination, attenuated and killed vaccines are the choices for swine industry. A study comparing commercial MLV and killed vaccines found that the MLV vaccine but not the killed one confers protective immunity in sows (32). It seems that cell-mediated immunity plays a role in the protective immunity. Therefore, development of an improved attenuated live vaccine to induce protective immunity against heterologous PRRSV strains should be pursued.

The infectious cDNA clones of the Lelystad virus, the prototype of Type 1 PRRSV (33) and VR-2332, the prototype of Type 2 PRRSV (34) were first established in 1998 and 2003, respectively. Reverse genetic technology has been applied to many other PRRSV strains since then (35-45). This technology allows for alteration of the cDNA of the PRRSV genome and generation of mutant viruses to examine viral virulence traits and to promote vaccine development.

Objectives: From your research proposal.

1) Construct an infectious clone of A2MC2 passage 90 virus that still induces IFN production.

We will complete sequencing of A2MC2 P90 to identify its genomic variations from the wild type virus. An infectious cDNA clone of A2MC2 P90 virus will be constructed, which will enable the manipulation of the P90 genome for gene functional studies and future vaccine development. The growth properties, such as multi-step growth curve, plaque size, and IFN induction of the rescued viruses will be characterized.

2) Assess the A2MC2 high passages in elicitation of the host immune response.

We hypothesize that the A2MC2 high passages will still induce higher level of virus-neutralizing antibodies and cell-mediated immune response than the MLV. Under this objective, A2MC2 P70, P80, and P90 viruses will be used to determine their capacity of eliciting the host immune response in pigs in comparison with the wild type A2MC2 and MLV strains. Lung pathology will be evaluated for virulence assessment.

Materials & Methods: This section should include experimental design, methods and procedures used, number of animals, etc.

Cells and viruses

MARC-145 (46) and Vero (ATCC CCL-81) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). CRL-2843 (porcine macrophages, ATCC) were cultured in RPMI1640 medium supplemented with 10% FBS. Primary PAM cells were prepared from 4-8-week-old piglets and cultured in RPMI1640 medium supplemented with 10% FBS (47).

PRRSV strain A2MC2, VR-2385 and Ingelvac PRRS® MLV were propagated and titrated in MARC-145 cells. Virus yields were titrated by 10-fold serial dilutions and presented as the median tissue culture infectious dose ($TCID_{50}$) (48). Newcastle disease virus (NDV) strain LaSota carrying the gene of green fluorescence protein (NDV-GFP) was propagated and titrated in Vero cells (49).

Interferon bioassay

Detection of presence of IFNs in culture supernatant from PRRSV-infected MARC-145 cells was done as previously described (29). Briefly, the supernatant was diluted in DMEM and used to treat Vero cells in 96-well plates overnight, followed by inoculation with NDV-GFP. Fluorescence microscopy was conducted 24 h after NDV inoculation to observe GFP-positive cells.

Immunofluorescence assay (IFA)

PRRSV propagation in MARC-145 cells was detected with IFA using an N-specific monoclonal antibody EF11 (50). The infected cells in 96-well plate were fixed and rinsed with phosphate-buffered saline (PBS) pH7.2 before addition of the EF11 antibody. DyLight™ 488 conjugated goat anti-mouse IgG (Rockland Immunochemicals Inc., Limerick, PA) was used to detect the EF11 binding to the N protein in the infected cells. Observation of N-positive cells was conducted under fluorescence microscopy.

Western blotting

Total proteins in cell lysate samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to nitrocellulose membrane (51). Blotting of the membrane with antibodies against RIG-I (Santa Cruz Biotechnology, Inc., Dallas, TX) and tubulin (Sigma-Aldrich Corp, St. Louis, MO) was conducted. Horseradish peroxidase-conjugated secondary antibodies (Rockland Immunochemicals Inc.) and chemiluminescence substrate were used to reveal specific reactions by the primary antibodies. Chemi-Doc Imaging System (Bio-Rad, Hercules, CA) was used to capture the luminescence signal.

RNA isolation, reverse transcription, and real-time PCR

Total RNA was isolated with the TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA) following the manufacturer's instructions. Reverse transcription followed by PCR (RT-PCR) and real-time PCR were conducted to amplify target PRRSV sequences or to determine PRRSV RNA level (47, 52). Detection of ribosomal protein L32 (RPL32) expression in the same sample was conducted to normalize the total input RNA. Primers of real-time PCR in this study previously described (26) and

analysis of relative transcript levels were performed by normalization of RPL32 in comparison with controls.

For RT-PCR to determine possible deletions in the nsp2 region of the A2MC2 genome during high passages, primers 85nspF3 (5'CTCGACGAACTCAAAGACC3') and 32nsp2R2 (5'CTGCGGACGGAGCTGATGTGC3') were used to amplify the target fragment with Phusion Flash High-Fidelity PCR Master Mix (Fisher Scientific, Pittsburgh, PA).

Plaque assay

A plaque assay in MARC-145 cells was done to compare the growth property of A2MC2 high passage with the wild type virus (29). Briefly, PRRSV A2MC2 was diluted to 10 and 100 $TCID_{50}$ per ml and added to the monolayer cells in 6-well plates at 1 ml per well. After 2 h incubation at 37°C, the inoculum was removed and 3 ml 0.5% agarose overlay containing the complete growth medium was added. The cells were stained at 72 h after incubation by addition of 2 ml neutral red mixture with agarose and observed for plaques after further overnight incubation.

Virus neutralization assay

Virus neutralization assay was performed on MARC-145 cells to determine PRRSV-neutralizing antibodies in pig serum samples (1). VR-2332, the prototype of type 2 PRRSV with nucleic acid identity of 99.8% to A2MC2 (29), was used as target virus in the assay at 100 $TCID_{50}$ for each reaction. The starting dilution of serum samples was 1:8, followed by 2-fold serial dilutions. IFA with N-specific monoclonal antibody was conducted 24 h after inoculation of the cells. 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.

Sequencing

RNA isolation from A2MC2 virions was done for reverse transcription, PCR amplification and DNA sequencing by chain-termination method using ABI Genetic Analyzer 3130 (ThermoFisher Scientific, Waltham, MA) (29). Sequence assembly and analysis was done with LaserGene Core Suite (DNASTAR Inc., Madison, WI). The cDNA sequence of the full-length A2MC2-P90 genome has been deposited in GenBank (accession number: KU318406).

Construction of cDNA clone of A2MC2-P90

PCR was done to amplify four fragments spanning the full-length cDNA of A2MC2-P90 genomic RNA. The unique restriction sites FseI, PmeI and BsrGI in the genome were used to assemble the cDNA clone. Fragment swapping was done to clone the cDNA into the target plasmid pIR-A2MC2, which contains a hammerhead ribozyme and a hepatitis delta virus ribozyme to the 5' end and 3' end of the cDNA. DNA sequencing was done to confirm the addition, orientation and correct sequence of the two ribozymes in the recombinant pCAGEN-A2P90-Rz. Correction of point mutations in the cDNA clone was done with Thermo Scientific™ Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific).

Animal study

Two animal studies were conducted after approval by Institutional Animal Care and Use Committees (IACUC) of the University of Maryland and Iowa State University according to relevant guidelines and policies for the care and use of laboratory animals. The first animal study was to determine the virulence of the high passages of A2MC2 virus. Three-week-old PRRSV-negative piglets were randomly divided into five groups with 4 pigs in each group. The piglets in groups 1 to 4 were inoculated with 1 ml of PRRSV strains A2MC2-P9, A2MC2-P75, A2MC2-P90, and Ingelvac PRRS® MLV, respectively, at 5 x 10^5 $TCID_{50}$/ml via intranasal (I.N.) inoculation, while group 5 was sham-infected with PBS pH7.2. The pigs in each group were euthanized on day 14 post infection (DPI14) by

pentobarbital overdose (FATAL-PLUS, Vortech Pharmaceuticals, LTD. Dearborn, MI). Visible macroscopic lung lesions and histopathology were scored and recorded as previously described (53, 54). 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 (TO, PGH). If results disagreed, they were combined and the average was used for further analysis.

The second animal study was conducted to assess the immunogenicity of high passages of A2MC2. Three-week-old PRRSV-negative piglets were randomly divided into four groups with 4 pigs in each group. The piglets in groups 1 to 4 were inoculated with 1 ml of PRRSV A2MC2-P9, A2MC2-P90, and Ingelvac PRRS® MLV, respectively, at 5 x 10^5 $TCID_{50}$/ml via intramuscular (I.M.) inoculation, while group 4 was mock-infected with PBS pH7.2. The I.M. route is generally used for porcine vaccination. Blood samples were collected weekly. The pigs were euthanized on DPI48. To assess the antibody response against PRRSV in the pigs, serum samples of DPI35 was tested with a commercial PRRSV ELISA kit (IDEXX PRRS X3 Ab Test; IDEXX Inc., Westbrook, MA, USA) according to the manufacturer's instructions. A sample-to-positive (S/P) ratio greater than 0.4 was considered positive.

Statistical analysis

Differences between treatment samples and control were assessed by the Student *t*-test. Differences between two groups for VN antibody titers of individual pigs were analyzed using analysis of variance (ANOVA). A two-tailed *P*-value of less than 0.05 was considered significant.

Results: Report your research results by objective.

Serial passaging of A2MC2 in MARC-145 cells

PRRSV strain A2MC2 was subjected to serial passaging in MARC-145 cells to minimize previously observed moderate virulence (1). The A2MC2 virus was passaged in MARC-145 cells for 90 consecutive passages. For each passage, the cells were frozen and thawed three times when cytopathic effect (CPE) occurred over 50% of the cells. Virus samples were collected for each passage. IFN bioassay results showed that treatment of Vero cells with the supernatnat of A2MC2 passage 90 (A2MC2-P90) even at a dilution of 1 to 16 inhibited the replication of NDV-GFP (Fig. 1A). This suggested that A2MC2-P90 retains the capacity of IFN induction of the wild type A2MC2.

To confirm IFN induction by strain A2MC2-P90, we performed immunoblotting detection of RIG-I, which is upregulated by type I IFNs (55). Result showed that the RIG-I level increased in the Vero cells treated with culture supernatant from A2MC2-P90-infected MARC-145 cells, whereas no change in RIG-I level was observed in cells treated with supernatant from VR-2385-infected cells (Fig.1B). Compared with treatment of mock-infected cells, the treatment with supernatant

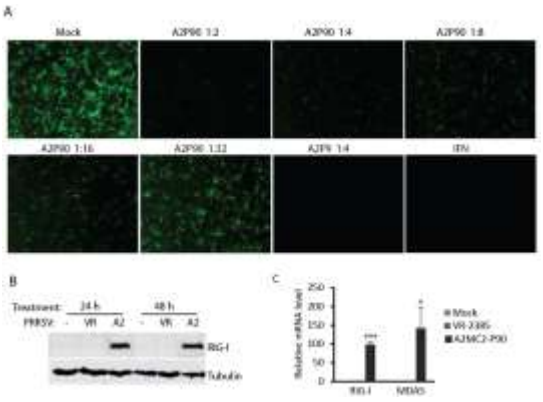

Fig. 1. A2MC2-P90 induces synthesis of interferons. A. Interferon bioassay in Vero cells. Dilutions of cell culture supernatant of MARC-145 cells infected with A2MC2-P90 (A2P90) or A2MC2-P9 (A2P9) were used to treat Vero cells. At 12 h after the treatment, the Vero cells were inoculated with NDV-GFP. B. RIG-I in Vero cells treated with culture supernatant from MARC-145 cells infected with VR-2385 (VR) or A2MC2-P90

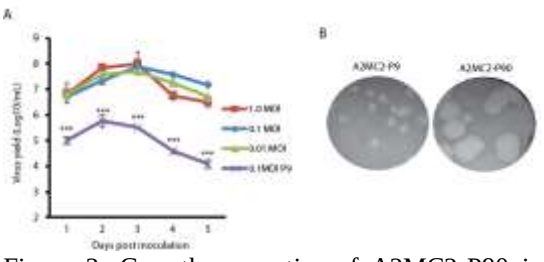

Figure 2. Growth properties of A2MC2-P90 in MARC-145 cells. A. Multi-step growth curve of A2MC2-P90 in MARC-145 cells. Virus yields at different time points after inoculation were titrated by an immunofluorescence assay. B. Plaque assay in MARC-145 cells.

from A2MC2-P90 infected cells led to 97.8 and 141.5-fold higher RIG-I and MDA5 transcript levels, respectively, whereas treatment with supernatant from VR-2385-infected cells had only 0.9 and 0.7-fold of RIG-I and MDA5 transcript levels, respectively (Fig. 1C).

Growth property determination and plaque assay

A2MC2-P90 was tested in MARC-145 cells for growth properties, including a multi-step growth curve and a plaque assay as described previously (29). The virus yields reached its peak at 72 hours post inoculation (hpi) and were 7.7, 7.9 and 8.0 Log_{10}/ml in $TCID_{50}$ for the cells with inoculum at an MOI (multiplicity of infection) of 0.01, 0.1 and 1, respectively (Fig. 2A). The virus yields for the cells inoculated with A2MC2-P90 at an MOI of 0.1 and harvested at 24, 48, 72, 96 and 120 hpi were 6.7, 7.3, 7.8, 7.6 and 7.2 Log_{10}/ml, respectively, which were significantly higher than the yields from the cells inoculated with wild type A2MC2 at an MOI of 0.1: 5.0, 5.7, 5.5, 4.6, and 4.1, respectively. Similar trends and titers of virus yields were observed for the samples harvested from the cells with the three different amounts of A2MC2-P90 inoculation.

A plaque assay was done for A2MC2-P90 and compared with wild type A2MC2. The plaque sizes of A2MC2-P90 were 8-10 mm in diameter, much bigger than the wild type A2MC2 palques, which were generally 3-4 mm in diameter (Fig. 2B). The larger size of plaques produced by A2MC2-P90 is consistent with its higher yield compared to the wild type virus.

Sequencing of cDNA of A2MC2-P90 genomic RNA

The virions of A2MC2-P90 were used for RNA isolation and RT-PCR. DNA sequencing of the PCR products was done and compared with sequences of wild type A2MC2 virus. Variations of nucleotides and derived amino acids in comparison with wild type A2MC2, VR-2332 and Ingelvac PRRS® MLV were identified. The locations of the differences in genomic RNA are illustrated in Fig. 3. The A2MC2-P90 genome has a deletion of 543 nucleotides (2994-3536) in ORF1a in comparison with wild type A2MC2 virus, leading to a deletion of 181 amino acid residues in hypervariable region of nsp2. Moreover, compared to the wild type, the A2MC2-P90 has 35 nucleotide mutations, among which 26 are non-synonymous, leading to 26 amino acid changes.

Fig. 3. Illustration of sequence variation of A2MC2-P90 (KU318406) in comparison to VR-2332 (U87392), Ingelvac PRRS® MLV (AF066183) and wild type A2MC2 (JQ087873). 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 in comparison with VR-2332 are indicated by vertical bars. The wide bars indicate the unique nucleotides identified earlier for A2MC2 in comparison with VR-2332 and MLV. Deletion (Del) in A2MC2-P90 from nt2994 to 3536 is indicated.

Interestingly, among the 15 unique nucleotides in A2MC2 genome compared with Ingelvac PRRS® MLV and VR-2332 (29), 14 remained the same in the A2MC2-P90 genome (Fig. 3). As a result, 5 of the 6 unique amino acid residues of A2MC2 compared to the MLV and VR-2332 remained the same in A2MC2-P90. The conserved five nucleotides leading to unique amino acids in A2MC2 are nt7621, 9655, 12012, 12972 and 12975 and the five unique residues are Ser20 in nsp8/9, Leu13 in nsp10, Gly135 in nsp12, and Val93 and Val94 in GP3. This result indicates that the 14 nucleotides in A2MC2 are highly conserved and sustained during the 90 serial passages. It also suggests that these 14 nucleotides or their related RNA structures might correlate with the feature of A2MC2 in IFN induction.

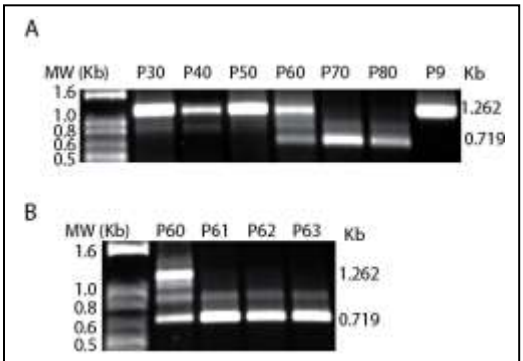

Fig. 4. Identification of the initial A2MC2 passage that carries the nsp2 deletion in genome. A. PCR detection of the nsp2 deletion in A2MC2 passages. The passages P30 to P80 were tested. B. Identification of the initial A2MC2 passage that has the nsp2 deletion.

The deletion in ORF1a occurs in passage 60 of A2MC2

Having noticed the deletion in nsp2 of A2MC2-P90, we wondered at which passage the deletion occurred. RT-PCR was conducted to amplify a fragment spanning the deletion area. The expected sizes of the PCR product are 719 bp for A2MC2-P90 and 1262 bp for wild type A2MC2. The PCR products for passage 30, 40 and 50 are the same size as wild type A2MC2, while the sizes of passage 70 and 80 are the same as A2MC2-P90 (Fig. 4A). There were two main bands in PCR products of passage 60. Therefore, the deletion likely occurred around passage 60. PCR amplification of passage 60 through 63 showed that the size shift from 1262 bp to 719 bp likely occurred from passage 60 to 61 (Fig. 4B). The size shift suggests that mutant virus with the spontaneous deletion appeared to become the main virus quickly.

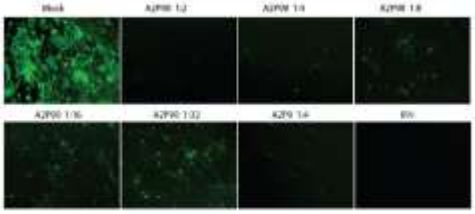

Fig. 5. A2MC2-P90 induces IFN synthesis in PAM cells. IFN bioassay is done in CRL2843 cells, in comparison with wild type A2MC2.

A2MC2-P90 induces interferons in PAM cells

PAMs are the major target cells for PRRSV infection *in vivo* (56). To determine if A2MC2-P90 can infect PAMs and induce interferons, we inoculated PAMs with the high-passage virus at an MOI of 3. Wild type A2MC2 was included as a control. Interferon bioassay was conducted on CRL-2843 cells, immortalized porcine macrophages that are not susceptible to PRRSV, as reported (29). Results showed that the supernatant of the A2MC2-P90 infected PAMs induced an antiviral effect in CRL-2843 cells by blocking the replication of NDV-GFP (Fig. 5). The supernatant dilutions at 1 to 32 still induced inhibition of NDV-GFP.

Construction of cDNA infectious clone of A2MC2-P90

A DNA-launched version of the cDNA infectious clone was constructed. Sequences of hammerhead ribozyme and hepatitis delta virus ribozyme were added at the 5' end and 3' end, respectively, of the cDNA of A2MC2. The full-length PRRSV sequence in pCAGEN-A2P90-Rz was subjected to DNA sequencing. Comparison with sequences of the parental A2MC2-P90 showed that the right sequence was cloned. The resulting plasmid pCAGEN-A2MC2-Rz was used to transfect MARC-145 cells. The transfected cells were harvested four days after transfection and supernatant of the cell lysate was passaged in MARC-145 cells. Characterization of the rescued virus is being undertaken.

Non-virulence of A2MC2-P90 in vivo

The objective of the serial passaging of A2MC2 was to attenuate the strain. To determine the degree of attenuation of the A2MC2-P90, we conducted an animal study by inoculating 3-week-old PRRSV-negative piglets. A2MC2-P9, A2MC2-P75, and Ingelvac PRRS® MLV were included in the animal study for control. Compared with pigs inoculated with A2MC2-P9, the pigs infected with A2MC2-P75 and A2MC2-P90 had significantly lower macroscopic lung lesion scores,

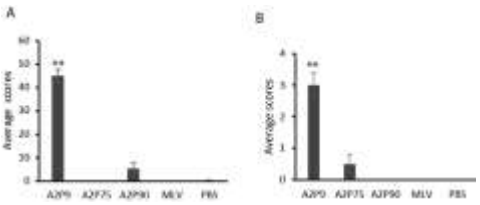

Fig. 6. Lung lesions in pigs infected with PRRSV A2MC2-P9, A2MC2-P75, A2MC2-P90 and MLV at 14 days post-infection (DPI). Four pigs from each group were necropsied. A. Average macroscopic lung lesion scores. A2: A2MC2. **B**. Average microscopic lung lesion scores. Significant differences between the group of A2MC2-

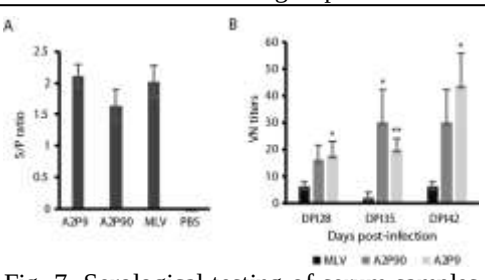

Fig. 7. Serological testing of serum samples from pig studies. Four pigs from each group were infected. A. ELISA of PRRSV antibodies in serum samples of 35 days post-infection (DPI). The S/P ratio above 0.4 is considered positive. B. Virus-neutralization assay against PRRSV VR-2332. The virus neutralization (VN) titers are shown as reciprocal of serum dilutions shown VN activity. Significant differences between the group of MLV-infected pigs are denoted by * and **, which indicate $P < 0.05$ and $P < 0.01$, respectively.

like the MLV-infected pigs and the mock-infected control in magnitude (Fig. 6A).

Microscopically, the interstitial pneumonia scores of the pigs infected with A2MC2-P75 and A2MC2-P90 were significantly lower than pigs infected with A2MC2-P9 (Fig. 6B). Both A2MC2-P75 and A2MC2-P90 groups had pathology scores similar to the MLV-infected or mock-infected pigs. All the groups except for A2MC2-P9 had no significant difference from the mock-infected control group. These results suggest that under the study conditions, A2MC2-P75 and A2MC2-P90 are avirulent in pigs, like the MLV strain.

A2MC2-P90 elicits higher level virus-neutralizing antibodies than the MLV strain

To assess the immunogenicity of A2MC2-P90, we conducted an animal study by inoculating 3-week-old PRRSV-negative piglets with A2MC2-P9, A2MC2-P90, and Ingelvac PRRS® MLV viruses. ELISA result showed that all pigs that were inoculated with the PRRSV viruses developed specific antibodies by DPI35, whereas the pigs of mock-infected group were all negative (Fig. 7A). The average S/P ratios for the virus-infected groups were over 1.6 for all groups without much difference.

Virus-neutralizing antibody assay was conducted for serum samples of DPI28 to DPI42 based on our previous study showing the appearance of VN antibody at DPI28 (1). All pigs in groups of A2MC2-P9 and A2MC2-P90, and three of four in MLV group had detectable VN antibodies at DPI28 (Fig. 7B). The average VN titers of the A2MC2-P90 and A2MC2-P9 groups were higher than those in MLV group for DPI28, DPI35 and DPI42 samples. The results show that similar to the wild type A2MC2 virus, A2MC2-P90 elicits higher VN antibodies than the MLV strain in this study.

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

Interferon induction is a unique characteristic of PRRSV strain A2MC2 as PRRSV strains generally antagonize interferon synthesis (29, 57). Considering the importance of interferons in activating the adaptive immune response, this feature may be desired in vaccine development against PRRS. Remarkably, the capability of strain A2MC2 to induce interferons is sustained after 90 serial passages in MARC-145 cells. Like the wild type virus, the high-passage virus also induces interferons in PAM cells. Moreover, the A2MC2-P90 is attenuated shown by its non-virulence in pigs and elicits higher virus-neutralizing antibodies.

Sequence comparison showed that 14 of 15 unique nucleotides of A2MC2 in comparison with both VR-2332 and MLV (29) are conserved in the A2MC2-P90 genome. Among the six unique amino acid residues, five are identical in both wild type A2MC2 and A2MC2-P90 and are located in nsp8/9, nsp10, nsp12 and GP3. These residues do not correlate with virulence as the A2MC2-P90 is avirulent. The nsp8 has unknown functions. The nsp9 is the RNA-dependent RNA polymerase; nsp10 is the helicase and GP3 is a structural glycoprotein (58, 59). The nsp12 induces STAT1 phosphorylation at Ser727 and may contribute to expression of inflammatory genes (60). None of these genes are known to be involved in the PRRSV antagonizing feature of interferon induction. It is not known whether these proteins or the nucleotide-related RNA structures play a role in inducing interferon induction. Further studies are needed to address the question.

Compared to the wild type virus, A2MC2-P90 genome has a deletion of 543 nucleotides in nsp2. The deletion likely occurred around passage 60 as there were both 719 and 1262 bp bands in the PCR of passage 60, but the 1262 bp band disappeared in passage 61. It appears that the virus with deletion grows faster and quickly became the main virus in passage 61. A2MC2-P90 propagates faster with higher yield in MARC-145 cells than the wild type A2MC2. Our results are consistent with an earlier

report that the spontaneous nsp2 deletion contributes to the faster virus propagation in a different strain *in vitro* (41). However, the nsp2 deletion does not have an effect on PRRSV virulence for that strain *in vivo.*

In addition, A2MC2-P90 has 35 nucleotide differences compared to the wild type virus, scattered throughout the genome. Nsp9 and nsp10 were found to contribute to fatal virulence of high pathogenic PRRSV strains in China (61). There are one and three different amino acid residues in nsp9 and nsp10, respectively, between A2MC2-P90 and the wild type virus (Table 1). Compared with moderate virulent VR-2332 and its derived avirulent MLV, these residues in the wild type A2MC2 are not unique (supplemental Table 1), suggesting that their correlation with virulence is unlikely. There is no unique synonymous mutations in nsp9 and nsp10 in A2MC2-P90. It is thus unknown which nucleotide mutations contribute to the attenuation of A2MC2, possibly a combined effect of the multiple mutations has to be considered. Among the 35 nucleotide mutations of A2MC2-P90 in comparison with the wild type A2MC2, only nt13011 is the same as in the MLV but different from VR-2332, leading to serine in both A2MC2-P90 and the MLV, and glycine in strains A2MC2 and VR-2332. The significance of this one amino acid variation between both moderate virulent strains and their avirulent descendants is not known and may need to be investigated.

A2MC2-P90 virus replicates faster in MARC-145 cells by inducing larger plaques and having higher titer of virus yield. It appears that A2MC2-P90 virus is less sensitive to the interferons it induces, as it replicates well when the cells are inoculated at an MOI of 1, while the wild type replicates poorly at this amount of inoculum (29). This indicates that A2MC2-P90 virus has been adapted to the cells and may gain the ability to dampen the interferon-activated antiviral response.

The animal studies demonstrate that A2MC2-P90 is avirulent and elicits better adaptive immune response than the MLV strain. The ELISA result showed that all PRRSV-infected pigs were seroconverted by DPI35. The VN test result showed most infected pigs had detectable VN antibodies by DPI28. The pigs infected with A2MC2-P90 had higher VN titers than the MLV group. The results indicate that the high passage of A2MC2 carries similar immunogenicity as the wild type virus.

In conclusion, the unique feature of interferon induction in both MARC-145 and PAM cells sustains the 90 serial passaging. A2MC2-P90 propagates more rigorously in MARC-145 cells than wild type A2MC2. A2MC2-P90 is avirulent in pigs. Sequence analysis shows A2MC2-P90 has a 543-nucleotide deletion in nsp2 and 35 nucleotide mutations throughout the genome in comparison to the wild type virus. A2MC2-P90 is avirulent and elicits higher VN antibodies than the Ingelvac PRRS® MLV strain. Further characterization of the attenuated virus is warranted for development of an improved vaccine against PRRS. Construction of the infectious clone of A2MC2-P90 will facilitate further study of the virus and vaccine development.

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