AASV Foundation Research Report – Final Report

Title: Comparison of PRRSV virus isolation in different cell lines towards improving success of isolating PRRSV from clinical samples

Authors/investigators: Jianqiang Zhang, Phillip Gauger, Karen Harmon, Rodger Main, Chong Wang. Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University.

Duration: June 1, 2017 through May 31, 2018

Final Report coverage: June 1, 2017 – May 31, 2018

Report

1. Statement of the problem

Porcine reproductive and respiratory syndrome (PRRS), characterized by reproductive failure in breeding females and respiratory distress in pigs of all ages, is an economically important disease globally. In a 2013 analysis, its economic impact on the U.S. swine industry was estimated at $664 million annually or $1.8 million per day (Holtkamp et al., 2013). The causative agent porcine reproductive and respiratory syndrome virus (PRRSV) includes at least 2 distinct genotypes: type 1 (PRRSV-1; European type) and type 2 (PRRSV-2; North American type), with extensive genetic variability between or even within these genotypes (Nelsen et al., 1999). For PRRSV-2, restriction fragment length polymorphism (RFLP) typing has been widely used to describe genetic diversity and genetic relatedness of viruses throughout North America but it has apparent drawbacks (Cha et al., 2004; Han et al., 2006; Wesley et al., 1998; Yoon et al., 2001). A phylogenetic lineage classification system (lineages 1 to 9) has been proposed for PRRSV-2 (Shi et al., 2010; Shi et al., 2013) to better classify global PRRSV-2 strains although this lineage system may be further refined.

Currently, PRRSV-specific real-time RT-PCRs and serological assays have been commonly used for the detection of PRRSV. However, PRRSV virus isolation (VI) is still frequently needed for various applications. For example, 1) PRRSV VI is conducted to determine if a sample contains infectious (live) virus. 2) Due to high rate of genetic and antigenic diversity, the commercial PRRSV vaccines are not always effective against field isolates. Swine veterinarians often request isolation of PRRSV for producing autogenous vaccines. 3) In order to further characterize some PRRSV strains (e.g. pathogenesis by inoculation of animals, virus-host cell interactions, etc.), cell culture isolates are needed. 4) Development and validation of some diagnostic assays need PRRSV cell culture isolates (e.g. virus-specific indirect fluorescent antibody [IFA] assay and virus-specific virus neutralization assay for antibody testing).

A MARC-145 cell line derived from the African monkey kidney cell line MA-104 is commonly used for isolating PRRSV (Kim et al., 1993). However, not all PRRSV strains

can grow in MARC-145 cells. Porcine alveolar macrophages (PAMs) are believed to be superior to simian cells for primary isolation of field PRRSV strains (de Abin et al., 2009) and are generally used to complement isolation of PRRSV in addition to MARC-145 cells. Nevertheless, preparation of reliable PAMs primary culture could be a challenge as only pigs of high health status and <8 weeks of age are recommended as the source of PAMs (Wensvoort et al., 1992). In addition, PAMs are primary cells and cannot be continuously passed and need to be prepared periodically. However, different batches of PAMs are not always equally susceptible to PRRSV; thus, verification of each batch of PAMs before use is needed. Due to these disadvantages, PAMs have not been routinely used by many veterinary diagnostic laboratories for PRRSV VI. The ZMAC cell line, derived from the lung lavages of porcine fetuses, was developed (Calzada-Nova et al., 2012) and has recently been made available to the public. However, ZMAC cells have not been evaluated for PRRSV VI using a large number of clinical samples.

In addition to cells used for VI, success of isolating PRRSV could also be affected by virus concentration in the samples as reflected by PRRSV PCR threshold cycle (C_T) values, by specimen types, and by PRRSV strains as reflected by different phylogenetic lineages. However, the impact of these factors on PRRSV VI has not been investigated in detail yet.

2. Objective(s)

- Compare isolation of PRRSV in MARC-145 and ZMAC cells using clinical samples.
- Evaluate the correlation of PRRSV concentration, genetic lineage, and specimen type to virus isolation success.
- Compare virus titers of isolates obtained in ZMAC and MARC-145 cells.
- Evaluate whether PRRSV isolated in ZMAC cells can grow in MARC-145 cells and vice versa.

3. Brief materials and methods

Nucleic acid extraction. Viral nucleic acids were extracted from clinical samples using a MagMAX Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, MA) and a Kingfisher Flex instrument (Thermo Fisher Scientific) following the instructions of the manufacturer.

PRRSV real-time RT-PCR. The commercial PRRSV real-time RT-PCR, VetMAX™ PRRSV NA&EU Reagent (Thermo Fisher Scientific), was used to test samples for the presence or absence of PRRSV RNA according to the manufacturer's instructions on ABI 7500 Fast instrument (Thermo Fisher Scientific). Threshold cycle (C_T) <37 was considered positive and $C_T \geq 37$ was considered negative.

Clinical samples. A total of 334 clinical samples were used and these included 264 PRRSV-2 PCR-positive clinical samples with various C_T ranges (109 serum samples with C_T 13.2-36.2; 96 lung samples with C_T 13-30.2; and 59 oral fluid samples with C_T 22.0-34.0) and 70 PRRSV-1 PCR-positive clinical samples with various C_T ranges (31 serum samples with C_T 18.4-36.8; 8 lung samples with C_T 21.6-35.4; and 31 oral fluid samples with C_T 28.0-36.8). These samples were selected from diagnostic cases submitted to the ISU VDL and used for virus isolation attempts in ZMAC and MARC-145 cells in this study.

Cells. MARC-145 cell line is a clone of the African monkey kidney cell line MA-104 (Kim et al., 1993). MARC-145 cells were cultured in the regular RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mg/ml gentamicin, 100 unit/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. The ZMAC cell line was derived from the lung lavages of porcine fetuses (Calzada-Nova et al., 2012) and was provided by Dr. Zuckermann, University of Illinois, Urbana, IL to the Aptimmune Biologics where we obtained this cell line. ZMAC cells were cultured in suspension in the RPMI-1640 medium with L-glutamine & 25 mM HEPES supplemented with MEM non-essential amino acids, 4 mM sodium pyruvate, 2 mM L-glutamine, 0.81% glucose, 10% fetal bovine serum, 0.01 µg/ml mouse colony stimulating factor (MCSF), 0.05 mg/ml gentamicin, 100 unit/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. MARC-145 and ZMAC cells were maintained at 37^{o}C incubator with 5% CO_2.

Virus isolation. For lung samples, 10% lung homogenates were first prepared. Then serum, lung homogenate, and oral fluid samples were filtered through 0.45 µM membranes to sterilize samples. Virus isolation was conducted in 24-well plates containing monolayers of MARC-145 cells and ZMAC cells, respectively. For each batch of samples, inoculation into two cell lines was conducted on the same day to avoid additional freeze-thaw of samples. For inoculation, 200 µl of samples were inoculated per well. After incubation for 1 h at 37°C incubator with 5% CO_2, inoculum was removed and 1 ml of the culture medium was added per well. The inoculated MARC-145 cells were checked daily for development of cytopathic effects (CPE) up to 5 days and the inoculated ZMAC cells were observed for 3 days. Then, cell culture supernatants were harvested and the cell plates were fixed with 80% cold acetone for 10 min at room temperature followed by immunofluorescence staining. If immunofluorescence staining is unclear, the supernatants were tested by PRRSV real-time RT-PCR to confirm VI results. If the first passage (P0) was VI negative, the P0 supernatants were inoculated into the respective cells for the second passage. Virus isolation was considered negative if the second passage was still negative.

Immunofluorescence staining. For MARC-145 cells, immunofluorescence staining was performed by incubating cells with PRRSV nucleocapsid protein-specific monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) (Rural Technologies, Inc., Brookings, SD) for 1 h at 37°C. The antibody conjugates were decanted and the cell plates were washed with phosphate buffered saline (PBS, 1x pH 7.4) for 3 times, 5 minutes per time. Plates were read under an Olympus IX71 fluorescent microscope (Olympus America Inc., Center Valley, PA) and results recorded. ZMAC cells were incubated with PRRSV nucleocapsid protein-specific unconjugated monoclonal antibodies (Rural Technologies, Inc., Brookings, SD) for 45 min at 37°C. The antibody was decanted and the cell plates were washed with phosphate buffered saline (PBS, 1x pH 7.4) for 3 times, 5 minutes per time. Then, the cell plates were incubated with a goat anti-mouse secondary antibody conjugated with FITC for 45 min at 37°C. After washed with PBS (3 times, 5 minutes each time), the plates were read under a fluorescent microscope as described above.

PRRSV ORF5 sequencing by Sanger method and phylogenetic analyses. PRRSV-2 ORF5 sequencing was performed using the Sanger method following the procedures that have been

previously described in detail (Zhang et al., 2017). The PRRSV-2 ORF5 sequences determined on the samples in this study together with the reference sequences representing the nine lineages of PRRSV-2 (Shi et al., 2010; Shi et al., 2013) were aligned using the MEGA6 MUSCLE program and the maximum likelihood phylogenetic tree was generated using the MEGA6 software (Tamura et al., 2013).

Virus titration. Twenty PRRSV-2 PCR-positive samples that were VI positive in both MARC-145 and ZMAC cells were selected for virus titer comparisons. Basically, 20 virus isolates obtained in MARC-145 cells were titrated in MARC-145 cells and 20 virus isolates obtained in ZMAC cells were titrated in ZMAC cells. Briefly, each virus isolate was serially 10-fold diluted and inoculated into MARC-145 cells or ZMAC cells grown in 96-well plates, 100 µl per well, five replicate wells per dilution. The plates were incubated at 37°C with 5% CO_2 for 3-5 days. Viral CPE was recorded daily. The cell culture supernatants were harvested and the cell plates were fixed with 80% cold acetone for 10 min at room temperature. Immunofluorescence staining on the fixed cells was conducted as described above. If immunofluorescence staining is unclear, the supernatants were tested by PRRSV real-time RT-PCR. The virus titers were calculated according to the method described by Reed and Muench (Reed and Muench, 1938) and expressed as $TCID_{50}$/ml.

Virus growth cross-checking. Seventy-eight PRRSV-2 isolates obtained in ZMAC cells were inoculated into MARC-145 cells to evaluate their growth. Similarly, 42 PRRSV-2 isolates obtained in MARC-145 cells were inoculated into ZMAC cells to evaluate their growth. Thirteen PRRSV-1 isolates obtained in ZMAC cells were inoculated into MARC-145 cells and one PRRSV-1 isolate obtained in MARC-145 cells was inoculated into ZMAC cells to evaluate their growth.

Statistical analyses. McNemar's test was performed to investigate the effects of cell lines or lineage classification on PRRSV VI outcomes. The effects of C_T values and specimen types on PRRSV VI were evaluated by Penalized Maximum Likelihood Estimates. Fisher's exact test was performed to examine the results of virus growth cross-checking.

4. Significant results

Comparison of PRRSV-1 VI from clinical samples in ZMAC and MARC-145 cells

Seventy PRRSV-1 PCR-positive clinical samples (31 serum, 8 lung, and 31 oral fluid samples) were used for VI attempts. None of the 31 PRRSV-1 oral fluid samples was VI positive in either ZMAC or MARC-145 cells (Table 1). Among the PRRSV-1 serum and lung samples, 25.8% (8/31) and 0% (0/31) of serum samples, 62.5% (5/8) and 12.5% (1/8) of lung samples, and 33.3% (13/39) and 2.6% (1/39) of serum plus lung samples, were VI positive in ZMAC and MARC-145 cells, respectively (Table 1). The 2 × 2 table of PRRSV-1 VI outcomes in ZMAC and MARC-145 cells are summarized in Table 2. The only one PRRSV-1 sample that was VI positive in MARC-145 cells was also VI positive in ZMAC cells. Out of the 13 PRRSV-1 samples that were VI positive in ZMAC cells, 1 was VI positive and 12 were VI negative in MARC-145 cells (Table 2). The PRRSV-1 VI success rates were significantly higher in ZMAC than in MARC-145 cells for serum (p = 0.0047), lung (p = 0.0455), and serum plus lung (p = 0.0005).

The success rates of isolating PRRSV-1 from serum and lung samples with different C_T values are summarized in Table 3. However, due to small number of PRRSV-1 samples, effects of PRRSV-1 C_T values on the VI outcomes were not statistically analyzed.

Comparison of PRRSV-2 VI from clinical samples in ZMAC and MARC-145 cells

Among the 264 PRRSV-2 samples, 47.7% (52/109) and 9.2% (10/109) of serum samples, 68.8% (66/96) and 45.8% (44/96) of lung samples, and 0% (0/59) and 0% (0/59) of oral fluid samples, were VI positive in ZMAC and MARC-145 cells, respectively (Table 1). When both serum and lung samples were combined, the success rates of PRRSV-2 VI in ZMAC and MARC-145 cells were 57.6% (118/205) and 26.3% (54/205), respectively.

The 2 × 2 table of PRRSV-2 VI outcomes in ZMAC and MARC-145 cells are summarized in Table 2. For PRRSV-2 VI, 10 serum and 37 lung samples were positive in both ZMAC and MARC-145 cells. However, among 52 serum and 66 lung samples that were VI positive in ZMAC cells, 42 serum and 29 lung samples were VI negative in MARC-145 cells. When combined together, 47 serum and lung samples were VI positive in both ZMAC and MARC-145 cells while 71 serum and lung samples were VI positive in ZMAC cells but negative in MARC-145 cells.

Statistical analyses indicated that the PRRSV-2 VI success rates were significantly higher in ZMAC than in MARC-145 cells for serum ($p < 0.0001$), lung ($p = 0.0002$), and serum plus lung ($p < 0.0001$). In addition, the specimen types had significant impacts on PRRSV-2 VI outcomes. The PRRSV-2 VI success rates were significantly higher in lung than in serum samples in either MARC-145 ($p < 0.0001$) or ZMAC cell line ($p =0.0028$).

Impact of C_T values on PRRSV-2 VI outcomes

The success rates of isolating PRRSV-2 from serum and lung samples with different C_T ranges are summarized in Table 3. The success rates of PRRSV-2 VI from serum and lung samples with different C_T values were 90.6% and 49.1% (C_T<20), 71.2% and 32.9% (C_T 20-25), 31.5% and 7.4% (C_T 25-30), 5.6% and 0% (C_T 30-33) in ZMAC and MARC-145 cells, respectively. The trend is clear that the success rates of PRRSV-2 VI decreased in serum and lung samples with increasing C_T values. Evidently, PRRSV-2 VI success rate was very low for samples with C_T 30-33 and there was no VI success from samples with C_T >33 in either ZMAC or MARC-145 cells. Statistical analyses based on the Penalized Maximum Likelihood Estimates method indicated that C_T values significantly affected the success rates of PRRSV-2 VI in MARC-145 cells (serum: $p = 0.0027$; lung: $p = 0.0003$; serum plus lung: $p < 0.0001$) and in ZMAC cells (serum: $p < 0.0001$; lung: $p < 0.0001$; serum plus lung: $p < 0.0001$).

Impact of genetic lineages on PRRSV-2 VI outcomes

A total of 264 PRRSV-2 clinical samples (109 serum, 96 lung, and 59 oral fluid samples) were used for VI attempts in this study. Due to unavailability of some clinical samples (very limited volume) and failure of ORF5 sequencing on some clinical samples, ORF5 sequences were eventually obtained from 220 PRRSV-2 samples and these included 91 serum, 85 lung, and 44 oral fluid samples. Phylogenetic analyses revealed that these samples contained PRRSV-2 belonging to lineage 1 (102 samples including 48 serum, 33

lung and 21 oral fluid samples), lineage 5 (69 samples including 27 serum, 30 lung and 12 oral fluid samples), lineage 8 (44 samples including 16 serum, 17 lung and 11 oral fluid samples), and lineage 9 (5 lung samples). Since PRRSV-2 VI was negative from all oral fluid samples, only 176 serum and lung samples with lineage information were further analyzed. PRRSV-2 VI outcomes on serum and lung samples classified into different lineages are summarized in Table 4. Only a small number (n=5) of samples included PRRSV-2 lineage 9 and, hence, they were excluded from the statistical analyses. For lineage 1 and lineage 8 samples (serum plus lung), the VI success rates were significantly higher in ZMAC cells than in MARC-145 cells ($p < 0.0001$ for lineage 1 and $p = 0.0039$ for lineage 8). In contrast, for the lineage 5 serum plus lung samples, the VI success rate between ZMAC and MARC-145 cells was not significantly different ($p = 0.3173$).

Comparison of virus titers of PRRSV-2 isolates obtained in ZMAC and MARC-145 cells
In order to compare the infectious titers of viruses isolated in ZMAC and MARC-145 cells, 23 PRRSV-2 isolates obtained in ZMAC cells were titrated in ZMAC cells and 23 PRRSV-2 isolates obtained in MARC-145 cells were titrated in MARC-145 cells; these virus isolates were derived from the same set of 23 clinical samples. As shown in Figure 1, fourteen out of 23 isolates obtained in ZMAC and MARC-145 cells had similar infectious titers whereas 6 ZMAC isolates had higher titers than MARC-145 isolates and 3 ZMAC isolates had lower titer than MARC-145 isolates. For three lineage 1 isolates with the 1-7-4 RFLP pattern, two had higher titers in MARC-145 cells and one had higher titer in ZMAC cells. For three lineage 1 isolates with the 1-8-4 RFLP pattern, they had higher titers in ZMAC cells. For nine lineage 5 isolates with the 2-5-2 RFLP pattern, seven had similar titers in the two cell lines, one had higher titer in MARC-145 cells and one had higher titer in ZMAC cells. For six lineage 8 isolates with the 1-3-2 RFLP pattern, they had similar titers in the two cell lines. Due to small numbers, statistical analyses were not conducted.

Cross-checking of PRRSV isolates growth in ZMAC and MARC-145 cells
In order to determine whether isolates derived from ZMAC cells can grow in MARC-145 cells and vice versa, 78 PRRSV-2 isolates obtained in ZMAC cells were inoculated into MARC-145 cells and 42 PRRSV-2 isolates obtained in MARC-145 cells were inoculated into ZMAC cells to evaluate their growth. The data are summarized in Table 5.

Among the 78 PRRSV-2 isolates obtained in ZMAC cells, 45 (57.7%) grew and 33 (42.3%) did not grow in MARC-145 cells. Interestingly, out of the 45 ZMAC isolates that grew in MARC-145 cells, 31 original samples were VI positive and 14 original samples were VI negative in MARC-145 cells. Among the 33 ZMAC isolates that did not grow in MARC-145 cells, 4 original samples were VI positive and 29 original samples were VI negative in MARC-145 cells. The genetic lineage information of PRRSV-2 isolates in each category was provided in Table 5 as well. The 78 PRRSV-2 isolates obtained in ZMAC cells included 33 lineage 1 isolates, 18 lineage 5 isolates, 18 lineage 8 isolates, 2 lineage 9 isolates, and 7 isolates with sequences unavailable. Among them, 12/33 lineage 1 isolates, 18/18 lineage 5 isolates, 10/18 lineage 8 isolates, and 2/2 lineage 9 isolates obtained in ZMAC cells grew in MARC-145 cells. Statistical analysis using Fisher's Exact Test indicated that there was a significant difference ($p = 0.0001$) between different genetic

lineages of ZMAC-derived PRRSV-2 isolates regarding their capability to grow in MARC-145 cells.

All of the 42 PRRSV-2 isolates obtained in MARC-145 cells grew in ZMAC cells. Among them, 35 original samples were VI positive and 7 original samples were VI negative in ZMAC cells. The 42 PRRSV-2 isolates obtained in MARC-145 cells included 10 lineage 1 isolates, 22 lineage 5 isolates, 9 lineage 8 isolates, and 1 lineage 9 isolate.

When the 13 PRRSV-1 isolates obtained in ZMAC cells were inoculated into MARC-145 cells, 4 (30.8%) grew and 9 (69.2%) did not grow in MARC-145 cells. When one PRRSV-1 isolate obtained in MARC-145 cells was inoculated into ZMAC cells, it grew.

5. Discussion of how results can be applied by practitioners

This study clearly demonstrates that ZMAC cells can significantly improve PRRSV VI from clinical serum and lung samples. The data about the impacts of PRRSV-2 concentration (C_T values), genetic lineages, and specimen types on VI outcomes can directly provide some guidance to the swine veterinarians, producers, and researchers to better serve their needs of obtaining PRRSV isolates for further characterization or for producing autogenous vaccines. Some specific points include:

- PRRSV-1 and PRRSV-2 was not isolated from any oral fluid samples evaluated in this study regardless of using ZMAC or MARC-145 cells, indicating that PRRSV VI from oral fluid is still a big challenge.
- For clinical serum and/or lung samples, the success rates of PRRSV-2 and PRRSV-1 VI were significantly higher in ZMAC cells than in MARC-145 cells. When PRRSV VI is negative in MARC-145 cells, PRRSV VI attempts in ZMAC cells should be explored.
- When serum and lung samples were compared for PRRSV-2 VI outcomes, the PRRSV-2 VI success rates were significantly higher in lung than in serum samples in either MARC-145 ($p < 0.0001$) or ZMAC cell line ($p = 0.0028$). The preferred specimen types for PRRSV VI are lung > serum > oral fluid.
- For serum and lung samples, the success rates of PRRSV-2 VI decreased with increasing C_T values. Evidently, PRRSV-2 VI success rate was very low for serum and lung samples with C_T 30-33 and there was no VI success from samples with C_T >33 either in ZMAC or MARC-145 cells. For cost effectiveness, it is not recommended to conduct PRRSV VI in clinical samples with C_T >30.
- The impacts of PRRSV-2 genetic lineages on VI varied. For lineage 1 and lineage 8 samples (serum plus lung), the VI success rates were significantly higher in ZMAC cells than in MARC-145 cells ($p < 0.0001$ for lineage 1 and $p = 0.0039$ for lineage 8). In contrast, for the lineage 5 serum plus lung samples, the VI success rate between ZMAC and MARC-145 cells was not significantly different ($p = 0.5271$). If the PRRSV-2 genetic lineage information is unknown in a clinical sample, the VI attempts will overall give a better outcome in ZMAC cells than in MARC-145 cells.
- For the 23 PRRSV-2 isolates positive in both cell lines, 14 had similar virus titers, 6 had higher titers in ZMAC than in MARC-145, and 3 isolates had slightly higher titer

in MARC-145 than in ZMAC cells. That is, most of the PRRSV-2 isolates obtained in ZMAC cells and MARC-145 cells had similar infectious titers.

- Among the 78 ZMAC-derived PRRSV-2 isolates, 45 (57.7%) grew and 33 (42.3%) did not grow in MARC-145 cells. In contrast, all 42 PRRSV-2 isolates obtained in MARC-145 grew in ZMAC cells. Hence, it is noteworthy that not all of the PRRSV-2 isolates obtained in ZMAC cells grew in MARC-145 cells while all of the PRRSV-2 isolates obtained in MARC-145 cells grew in ZMAC cells under the conditions of this study. Currently, the majority of autogenous vaccine manufacturing companies/labs use MARC-145 cells for PRRSV propagation; they may need to consider introducing ZMAC cells to their labs if they want to use PRRSV isolates obtained in ZMAC cells by veterinary diagnostic laboratories.

6. References

Calzada-Nova, G., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2012. Effect of the host cell line on the vaccine efficacy of an attenuated porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 148, 116-125.

Cha, S.H., Chang, C.C., Yoon, K.J., 2004. Instability of the restriction fragment length polymorphism pattern of open reading frame 5 of porcine reproductive and respiratory syndrome virus during sequential pig-to-pig passages. J Clin Microbiol 42, 4462-4467.

de Abin, M.F., Spronk, G., Wagner, M., Fitzsimmons, M., Abrahante, J.E., Murtaugh, M.P., 2009. Comparative infection efficiency of Porcine reproductive and respiratory syndrome virus field isolates on MA104 cells and porcine alveolar macrophages. Can J Vet Res 73, 200-204.

Han, J., Wang, Y., Faaberg, K.S., 2006. Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. Virus Res 122, 175-182.

Holtkamp, D.J., Kliebenstein, J.B., Neumann, E.J., Zimmerman, J., Rotto, H., Yoder, T., Wang, C., Yeske, P., Mowrer, C., Haley, C., 2013. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. Journal of Swine Health and Production 21, 72-84.

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch Virol 133, 477-483.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J Virol 73, 270-280.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493-497.

Shi, M., Lam, T.T., Hon, C.C., Murtaugh, M.P., Davies, P.R., Hui, R.K., Li, J., Wong, L.T., Yip, C.W., Jiang, J.W., Leung, F.C., 2010. Phylogeny-based evolutionary,

demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. J Virol 84, 8700-8711.

Shi, M., Lemey, P., Singh Brar, M., Suchard, M.A., Murtaugh, M.P., Carman, S., D'Allaire, S., Delisle, B., Lambert, M.E., Gagnon, C.A., Ge, L., Qu, Y., Yoo, D., Holmes, E.C., Chi-Ching Leung, F., 2013. The spread of type 2 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in North America: a phylogeographic approach. Virology 447, 146-154.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30, 2725-2729.

Wensvoort, G., de Kluyver, E.P., Pol, J.M., Wagenaar, F., Moormann, R.J., Hulst, M.M., Bloemraad, R., den Besten, A., Zetstra, T., Terpstra, C., 1992. Lelystad virus, the cause of porcine epidemic abortion and respiratory syndrome: a review of mystery swine disease research at Lelystad. Vet Microbiol 33, 185-193.

Wesley, R.D., Mengeling, W.L., Lager, K.M., Clouser, D.F., Landgraf, J.G., Frey, M.L., 1998. Differentiation of a porcine reproductive and respiratory syndrome virus vaccine strain from North American field strains by restriction fragment length polymorphism analysis of ORF 5. J Vet Diagn Invest 10, 140-144.

Yoon, K.J., Chang, C.C., Zimmerman, J., Harmon, K., 2001. Genetic and antigenic stability of PRRS virus in pigs. Field and experimental prospectives. Adv Exp Med Biol 494, 25-30.

Zhang, J., Zheng, Y., Xia, X.Q., Chen, Q., Bade, S.A., Yoon, K.J., Harmon, K.M., Gauger, P.C., Main, R.G., Li, G., 2017. High-throughput whole genome sequencing of Porcine reproductive and respiratory syndrome virus from cell culture materials and clinical specimens using next-generation sequencing technology. J Vet Diagn Invest 29, 41-50.

Table 1. Outcomes of PRRSV virus isolation from clinical samples in ZMAC and MARC-145 cells

Type	**Specimen**	**Number**	**ZMAC VI +**	**MARC-145 VI +**
PRRSV-2	Serum	N=109	47.7% (52/109)	9.2% (10/109)
	Lung	N=96	68.8% (66/96)	45.8% (44/96)
	Oral fluid	N=59	0% (0/59)	0% (0/59)
	Total	***N=264***		
PRRSV-1	Serum	N=31	25.8% (8/31)	0% (0/31)
	Lung	N=8	62.5% (5/8)	12.5% (1/8)
	Oral fluid	N=31	0% (0/31)	0% (0/31)
	Total	***N=70***		

Table 2. Comparison of PRRSV virus isolation in MARC-145 and ZMAC cells on serum and lung samples

Type	Specimen		MARC-145 VI +	MARC-145 VI -	Total
PRRSV-2	Serum	ZMAC VI +	10	42	52
		ZMAC VI -	0	57	57
		Total	10	99	109
	Lung	ZMAC VI +	37	29	66
		ZMAC VI -	7	23	30
		Total	44	52	96
	Serum & Lung	ZMAC VI +	47	71	118
		ZMAC VI -	7	80	87
		Total	54	151	205
PRRSV-1	Serum	ZMAC VI +	0	8	8
		ZMAC VI -	0	23	23
		Total	0	31	31
	Lung	ZMAC VI +	1	4	5
		ZMAC VI -	0	3	3
		Total	1	7	8
	Serum & Lung	ZMAC VI +	1	12	13
		ZMAC VI -	0	26	26
		Total	1	38	39

Table 3. Distribution of PRRSV PCR CT values and virus isolation outcomes in ZMAC and MARC-145 cells based on specimen types

Type	C_T value	Serum			Lung			Serum & Lung		
		Total	ZMAC VI +	MARC-145 VI +	Total	ZMAC VI +	MARC-145 VI +	Total	ZMAC VI +	MARC-145 VI +
PRRSV-2	<20	22	19 (86.4%)	5 (22.7%)	31	29 (93.5%)	21 (67.7%)	53	48 (90.6%)	26 (49.1%)
	20-25	32	23 (71.9%)	5 (15.6%)	41	29 (70.7%)	19 (46.3%)	73	52 (71.2%)	24 (32.9%)
	25-30	31	9 (29.0%)	0	23	8 (34.8%)	4 (17.4%)	54	17 (31.5%)	4 (7.4%)
	30-33	17	1 (5.9%)	0	1	0	0	18	1 (5.6%)	0
	33-37	7	0	0	0	0	0	7	0	0
	Total	109	52 (47.7%)	10 (9.2%)	96	66 (68.8%)	44 (45.8%)	205	118 (57.6%)	54 (26.3%)
PRRSV-1	<20	1	0	0	0	0	0	1	0	0
	20-25	6	3 (50%)	0	5	5 (100.0%)	1 (25.0%)	11	8 (72.7%)	1 (9.1%)
	25-30	9	4 (44.4%)	0	1	0	0	10	4 (40.0%)	0
	30-33	11	1 (9.1%)	0	0	0	0	11	1 (9.1%)	0
	33-37	4	0	0	2	0	0	6	0	0
	Total	31	8 (25.8%)	0	8	5 (62.5%)	1 (12.5%)	39	13 (33.3%)	1 (2.6%)

Table 4. Virus isolation outcomes of different PRRSV-2 lineages in ZMAC and MARC-145 cells

Lineage	Serum			Lung			Serum & Lung		
	Total	**ZMAC VI+**	**MARC-145 VI+**	**Total**	**ZMAC VI+**	**MARC-145 VI+**	**Total**	**ZMAC VI+**	**MARC-145 VI+**
1	48	29 (60.4%)	5 (10.4%)	33	24 (72.7%)	8 (24.2%)	79	53 (65.4%)	13 (16.0%)
5	27	4 (14.8%)	2 (7.4%)	30	15 (50%)	20 (66.7%)	59	19 (33.3%)	22 (38.6%)
8	16	6 (37.5%)	1 (6.3%)	17	13 (76.5%)	8 (47.1%)	33	19 (57.6%)	9 (27.3%)
9	0	0	0	5	3 (60%)	2 (40%)	5	3 (60%)	2 (40%)
Total	91	39 (42.9%)	8 (8.8%)	85	55 (64.7%)	38 (44.7%)	176	94 (53.4%)	46 (26.1%)

Note: ORF5 sequencing was successfully determined on 220 PRRSV-2 PCR-positive specimens used for VI (91 serum, 85 lung, and 44 oral fluid samples). Since all oral fluid samples were virus isolation negative, only serum and lung samples are listed here.

Table 5. Cross-checking of PRRSV-2 isolates growth in ZMAC and MARC-145 cells

a. 78 PRRSV-2 isolates obtained in ZMAC cells --> Re-inoculated into MARC-145 cells

Initial VI in ZMAC	Initial VI in MARC-145	ZMAC isolates re-inoculated into MARC-145	Number of isolates (%)	Lineage*
Pos	Pos	Pos	31 (39.7%)	6L1 + 16L5 + 8L8 + 1L9
Pos	Neg	Pos	14 (18.0%)	6L1 + 2L5 + 2L8 + 1L9 + 3 no seq
Pos	Pos	Neg	4 (5.1%)	4L1
Pos	Neg	Neg	29 (37.2%)	17L1 + 8L8 + 4 no seq
Subtotal			78 (100%)	33L1 + 18L5 + 18L8 + 2L9 + 7 no seq

b. 42 PRRSV-2 isolates obtained in MARC-145 cells --> Re-inoculated into ZMAC cells

Initial VI in MARC-145	Initial VI in ZMAC	MARC-145 isolates re-inoculated into ZMAC	Number of isolates (%)	Lineage**
Pos	Pos	Pos	35 (83.3%)	10L1 + 16L5 + 8L8 + 1L9
Pos	Neg	Pos	7 (16.7%)	6L5 + 1L8
Pos	Pos	Neg	0	
Pos	Neg	Neg	0	
Subtotal			42 (100%)	10L1 + 22L5 + 9L8 + 1L9

Notes:

* The 78 PRRSV-2 isolates obtained in ZMAC cells included 33 lineage 1 isolates, 18 lineage 5 isolates, 18 lineage 8 isolates, 2 lineage 9 isolates, and 7 isolates with sequences unavailable. Among them, 12/33 lineage 1 isolates, 18/18 lineage 5 isolates, 10/18 lineage 8 isolates, and 2/2 lineage 9 isolates obtained in ZMAC cells grew in MARC-145 cells.

** The 42 PRRSV-2 isolates obtained in MARC-145 cells included 10 lineage 1 isolates, 22 lineage 5 isolates, 9 lineage 8 isolates, and 1 lineage 9 isolates. All of these isolates obtained in MARC-145 cells grew in ZMAC cells.

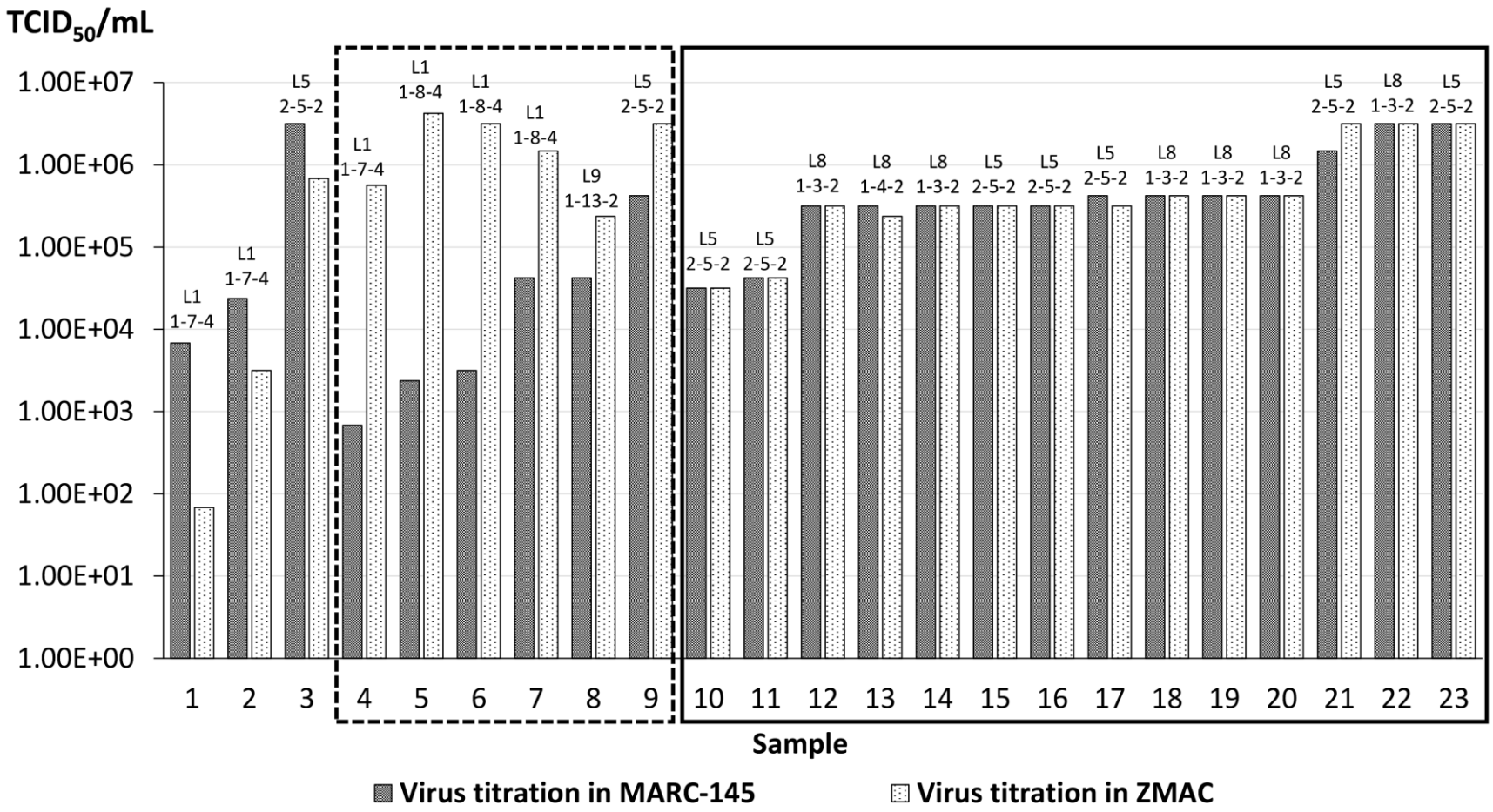

Figure 1. Comparison of infectious titers ($TCID_{50}$/ml) of 23 PRRSV-2 isolates obtained in ZMAC and MARC-145 cells. The same set of 23 clinical samples were VI positive in both ZMAC and MARC-145 cells. The isolates obtained in ZMAC cells were titrated in ZMAC cells and the isolates obtained in MARC-145 cells were titrated in MARC-145 cells. Six ZMAC isolates (#4-#9) having higher titers than MARC-145 cells are surrounded by dotted line. Fourteen ZMAC and MARC-145 isolates (#10-#23) having similar titers are surrounded by solid line. The genetic lineage and RFLP pattern of the isolates are labeled above the bars.