RESEARCH REPORT
SWINE HEALTH

Title: Genetic and antigenic characterization of a recent PRRSV isolate – **NPB #08-260**

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Date Submitted: May 29, 2010

Industry Summary

Porcine reproductive and respiratory syndrome virus (PRRSV) NC16845 was isolated from porcine tissue submitted to the Iowa State University Veterinary Diagnostic Laboratory in 2006. The isolate originated from twelve-week-old pigs from North Carolina exhibiting lethargy, coughing, respiratory distress and weight loss. The excessive mortality and morbidity was unusual and prompted further investigation of the PRRSV for unique genomic and growth characteristics. The objectives of this study were to (1) sequence the full genome of a recently isolated PRRSV designated NC16845 and (2) evaluate the *in vitro* growth characteristics compared to prototypes VR-2332 and MN184. Sequence analysis demonstrated that NC16845 was similar to atypical PRRSV JA142 although the NC16845 restriction fragment length polymorphism (RFLP) pattern is 1-18-2. One PRRSV sequence in the PRRSV database was found to be similar to NC16845 in the region of the attachment glycoprotein (ORF5) and was also isolated in North Carolina at approximately the same time. The NC16845 genome is 15,385 nucleotides in length, approximately 26 nucleotides shorter than the prototype VR-2332. Nucleotide degeneracy was noted in the replicase gene that corresponded to the highly variable non-structural protein 2 (nsp2) region similar to differences identified in the virulent strain PRRSV MN184. However, considerable nucleotide divergence was demonstrated between NC16845 and VR-2332 and MN184. NC16845 demonstrated slower replication characteristics in cell culture compared to VR-2332 and another virus isolated in the 1990's, VR2385; however, replication kinetics were somewhat similar to MN184. Plaque sizes were smaller compared VR-2332 but larger than those of MN184. These results emphasize the inverse correlation between *in vitro* growth characteristics and the *in vivo* virulence where highly virulent PRRSV isolates are less adapted to cell culture and exhibit a slower rate of replication and growth. The results indicate unique genomic and growth features of the novel PRRSV isolate NC16845. Consistent with previously characterized virulent PRRSV isolates, NC16845 contains a shorter genome with variations in specific regions that are reflective of a slower growth rate *in vitro*. In addition, NC16845 demonstrated the unique ability of PRRSV to alter its genome and adapt to selection pressure resulting in PRRSV isolates that may be more virulent and capable of circumventing prior herd immunity.

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Keywords. Porcine reproductive and respiratory syndrome virus, *in-vitro* analysis, virulence, sequence; growth rate.

Scientific Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is the cause of respiratory disease and reproductive failure in swine. The virus continues to have a significant economic impact on the swine industry in the United States and worldwide. PRRSV is an RNA virus and as such subject to variable rates of mutation and viral recombination. The emergence of novel, virulent strains of PRRSV in herds with prior immunity is not uncommon.

A virulent isolate of PRRSV, responsible for high morbidity and mortality, was isolated from a North Carolina swine farm in 2006. Affected pigs were twelve weeks old and demonstrated clinical signs of lethargy, coughing dyspnea and weight loss with elevated mortality. PRRSV was isolated from affected lung tissue submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). The PRRSV isolate, designated NC16845, was subsequently characterized through genomic sequencing and was evaluated for unique *in vitro* growth characteristics compared to three type 2 PRRSV isolates, which included the prototype VR-2332, MN184 and VR2385 isolated in the mid-90's.

The full length genome of NC16845 was found to be 15,385 nucleotides, which is similar to other type 2 PRRSV isolates that have been previously sequenced; however, restriction fragment length polymorphism (RFLP) analysis demonstrated a unique pattern designated 1-18-2. NC16845 shares an approximate nucleotide homology of 90.5% with atypical PRRSV JA142. Compared to VR-2332, nucleotide differences were identified in the ORF1a region known as non-structural protein 2 (nsp2) region. In addition, this region contained elevated nucleotide degeneracy and a discontinuous nucleotide deletion of 26 bases. Sequence homology with VR-2332 and MN184 was 88.2% and 77.3%, respectively.

NC16845 demonstrated slower replication in cell culture compared to VR-2332, MN184 and VR2385. NC16845 grew to a peak titer of $5.4x10^5$ plaque forming units per milliliter (PFU/ml) at 60 hrs post inoculation which was 4-13-fold lower than the growth of the other viruses. NC16845 was most similar in growth and replication properties to MN-184 PRRSV. Plaque assays resulted in plaques of intermediate size similar to VR2385, but larger than those of MN184 and smaller than the plaques induced by VR-2332. NC16845 plaques were clear and averaged 3.3 mm in diameter. Northern blots revealed NC16845 demonstrated a similar pattern of subgenomic RNA to MN184.

Collectively, these data indicate a slower replication rate and diminished growth properties of virulent PRRSV isolate NC16845 compared to prototype type 2 PRRSV strains. In addition, NC16845 contained fewer subgenomic RNA species similar to previously characterized MN184. The genome contains fewer nucleotide bases than VR-2332 and regions of heterogenous nucleotides with a discontinuous deletion that suggests that PRRSV NC16845 continues to evolve to eliminate dispensable regions of the genome.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, positive sense, enveloped, single-stranded RNA virus of significant economic importance in swine herds worldwide. PRRSV was first recognized in the Unites States in 1987 (Keffaber, 1989) and Europe in 1990 (Paton et al., 1991). The virus is ubiquitous in most swine populations. PRRSV is a member of the family *Arteriviridae* and the order *Nidovirales* (Cavanagh, 1997) which includes lactate dehydrogenase-elevating virus of mice, equine arteritis virus and simian hemorrhagic fever virus (Plagemann et al., 1992). Clinical manifestation of PRRSV includes systemic and respiratory disease in grow finish pigs and reproductive failure in naïve breeding animals (Halbur et al., 1996; Mengeling et al., 1998). PRRSV costs the US swine industry approximately $560 million per year due to production losses, treatment costs and excessive mortality.

PRRSV evolves rapidly through mutation resulting in the emergence of new, genetically diverse, strains (Pirzadeh et al., 1998; Rowland et al., 1999; Forsberg et al., 2001; Hanada et al., 2005). Recently, PRRSV isolates have emerged within the swine population with varying degrees of virulence (Nelsen et al., 1999; Han

et al., 2006; Fang et al., 2007) possibly resulting from a high degree of viral recombination allowing the virus to evade prior host immunity (Yuan et al., 1999; Yuan et al., 2000; Yuan et al., 2001; Yuan et al., 2004). In addition, the emergence of a virulent strain of PRRSV in Danish swine herds was attributed to a vaccine virus that reverted to virulence providing further evidence of PRRSV's innate ability to mutate (Madsen et al., 1998). Overall, the genetic and phenotypic diversity of PRRSV is increasing as there are over 13,000 different isolates within the PRRSV database to date (http://prrsvdb.org).

Genetic analysis has established two predominant PRRSV genotypes; the European (Type 1) and North American (Type 2) genotypes which share approximately 60% nucleotide identity (Allende et al., 1999; Nelsen et al., 1999). Historically, swine herds in the United States have been predominately being infected with Type 2 viruses; however, since 1999, Type 1 isolates have become more widely spread (Dewey et al., 2000; Fang et al., 2004). This genetic diversity has lead to the emergence of at least four genogroups of PRRSV in the U.S. based on their unique restriction fragment length polymorphism (RFLP) patterns. These viruses are of the Type 2 lineage and include: VR-2332, MN184, "atypical" PRRSV JA142 and a newly identified PRRSV with a 1-18-2 pattern.

Each full-length genome published offers more information concerning the structure of viral proteins and pinpoints regions of the genome associated with virulence. Open-reading-frame (ORF) 5, the standard ORF for PRRSV sequence analysis and tracking, was thought to be the most variable region of PRRSV. However, it is now well established that nonstructural protein 2 (nsp2), not usually sequenced, is much more variable. Therefore, additional sequencing outside of ORF5 has the ability to enhance our understanding of the molecular rules utilized by PRRSV.

Comparing more than 13,000 unique PRRSV sequences deposited in the PRRSV database, we have recently detected an apparently novel and virulent Type 2 PRRSV isolate designated NC16845. In this report, we examine PRRSV NC16845 in terms of sequence analysis, *in vitro* growth rate and plaque appearance and we compare these traits to prototype VR-2332 (1989 prototype). The complete sequence and the *in vitro* characteristics provide additional knowledge on the novel Type 2 PRRSV isolate.

Objectives

The main objective was to further characterize PRRSV isolate NC16845.

1. Sequence the full genome of NC16845.
2. Evaluate the growth kinetics of NC16845 through *in vitro* analysis and comparison to the prototype virus VR-2332.

Materials and Methods

PRRSV Isolates

PRRSV isolate NC16845 was recovered from a North Carolina farm in 2006; VR2385 PRRSV was recovered from an Iowa swine farm in 1995; and MN-184 was recovered from a Minnesota swine farm in 2001. The isolates were amplified by passage on MARC-145 cells. This procedure is well standardized and involves viral growth on defined EMEM medium containing 10% fetal bovine serum until PRRSV cytopathic effect (CPE) was visible (usually 2-4 days to reach 80% monolayer). At this time, the remaining infected cells were lysed by a freeze-thaw method followed by centrifugation at 2,000 x g for 10 min to pellet cell debris. The viral supernatant was distributed in 2-3 ml aliquots for titration and frozen at -80°C.

Full Genome Sequence Analysis

The complete genomic sequence was analyzed by comparing it to other standard PRRSV Type 2 strains. NC16845 was amplified twice on MARC-145 cells before sequencing. PRRSV RNA was extracted from infected cell supernatant using the QiaAmp RNA isolation kit (Qiagen). Purified RNA was amplified using SuperScript III One-Step RT-PCR Platinum Taq High Fidelity kit (Invitrogen) and universal PRRSV primers to generate 2.1–4.5 kb products. The genomic ends of the virus were generated using standard rapid amplification of cDNA Ends (5'- and 3'-RACE; Takara). PCR products were purified with QiaQuick kit (Qiagen) and

plasmids prepared using Topo TA cloning kit (Invitrogen). The poly-A tail with approximately 213 bases at the 5' end was sequenced using the 5'- RACE kit (Takara) and plasmid preparation using the Topo TA cloning kit. Resulting plasmids were transformed into *E. coli* and amplified. Amplified plasmids were then purified using QiaPrep Plasmid miniprep kit (Qiagen). PCR products (two-fold genome coverage) were submitted to the DNA Facility at the National Animal Disease Center for nucleotide sequence generation. Virus was purified and processed for deep sequencing according to an established protocol. Sequencing was conducted using the RNA virus sequencing genome sequencer 454 FLX system and assembly of multiple contigs generated by the instrument. Multiple regions of the genome that are not sequenced with the 454 FLX system required further primer design and traditional Sanger sequencing to span the regions/gaps between trace files where nucleotide sequence was presumably absent.

Viral Growth Assays

MARC-145 cell monolayers in T-25 flasks were infected with NC16845, VR2385, MN184 or VR-2332 viruses at a multiplicity of infection (MOI) of 0.01. After 1 h of attachment at room temperature with gentle mixing, the inocula were removed and the monolayers were washed three times with serum free EMEM. After washing, 7 ml complete medium was added and the flasks were then incubated for up to 5 days at 37°C in 5% CO_2. Samples were collected from culture supernatant at 12, 24, 36, 48, 60, 72, 96 and 108 hrs post infection (hpi) and titrated by viral plaque assay.

Plaque Analysis

Equal amounts of PRRSV-infected cell culture supernatants were diluted 10^3-10^6 fold in EMEM without FBS. Diluted virus was added to duplicate wells of a 6-well-plate that contained 90% confluent MARC-145 cell monolayers, followed by room temperature (RT) incubation for 1 hour with occasional redistribution of the inoculum. An equal mixture (5 ml total) of 2X EMEM medium and 2% SeaPlaque LE agarose (FMC) prewarmed at 60°C, was added to each well and left at room temperature for 15 min to solidify the agarose overlay. The cells were then transferred for incubation at 37°C in 5% CO_2 for 96-120 hours. Plaques were stained with crystal violet for permanent record in order to reveal their size and numbers. The results were recorded as the number of plaque forming units per milliliter of inoculum (PFU/ml). The morphology of representative plaques was recorded pictorially.

Northern Analysis

NC16845, VR2385, MN-184 and VR-2332 were inoculated in MARC-145 cells at an m.o.i. of 0.01. Two days after infection, total intracellular RNA was extracted using the Quiagen RNAeasy mini kit (Qiagen Inc., Valencia, CA) and electrophoresed
(15 µg/sample) on a glyoxal denaturing gel as previously described (Nelsen *et al.*, 1999). After electrophoresis, the RNA was transferred onto a 0.45 µm MagnaGraph nylon transfer membrane (MagnaGraph, GE, Osmonics) and crosslinked to the membrane (Yuan *et al.*, 2000). The membrane was probed with a digoxin-labeled (Roche-Applied Science, Indianapolis, IN) oligomer complementary to PRRSV 3'UTR. Post-hybridization, chemiluminescent detection was conducted using the DIG nucleic acid Detection Kit (Roche-Applied Science, Indianapolis, IN) according to manufacturer's instructions. Post-detection, membranes were exposed to X-ray film for approximately 10 minutes to visualize subgenomic RNA.

Results

Full genome sequencing.

A potential novel PRRSV, NC16845, was isolated from clinically affected swine on a North Carolina farm in 2006. Initial ORF5 nucleotide sequencing and comparison to the PRRSV database (>13,000 isolates; http://prrsvdb.org) revealed that the isolate was of North American Type 2 lineage. Interestingly, only one sequence in the PRRSV database, PRRSV0000008275, was similar with identical ORF5 amino acid sequences but two less nucleotide mutations. This PRRSV was also isolated in North Carolina in 2006 and was submitted

by the South Dakota State Diagnostic Laboratory. Restriction fragment length polymorphism (RFLP) analysis of the ORF5 genome also demonstrated this isolate belongs to the 1-18-2 pattern.

NC16845 genomic alignment of the ORF5 sequence with Type 1 (European) prototype strain Lelystad and EuroPRRSV revealed an approximate homology of 60.6% and 61.1%, respectively, on the nucleotide level. This confirmed that NC16845 was of Type 2 origin. The NC16845 genome was found to be 15,385 nucleotides in length without including the poly A tail. Surprisingly, genomic alignment with other Type 2 PRRSV revealed NC16845 demonstrated the highest similarity to prototype JA142 (RFLP pattern 1-4-2) and shared an approximate 90.5% homology on the nucleotide level. Alignment with prototype VR-2332 and MN184 demonstrated nucleotide homology of 88.2% and 77.3%, respectively. Table 1 presents the nucleotide comparison between full-length NC16845 and three additional PRRSV.

Table 1. Analysis of individual PRRSV genomic regions, degenerate bases and nucleotide comparison with prototype PRRSV.

NC16845 genomic analysis				% Nucleotide homology		
Region	Bases	Nucleotide length	Degenerate bases #	JA142	VR-2332	MN184
5'-UTR	1-192	192	6	98.4	92.7	86.7
ORF1A	193-7674	7482	91	88.6	85.5	78.7
ORF1B	7671-12039	4369	0	93.3	90.3	88.9
ORF2A	12045-12817	773	0	92.7	91.5	91.8
ORF2B	12060-12251	192	0	92.8	NA	93.9
ORF3	12670-13434	765	0	91.8	92.3	90.5
ORF4	13215-13751	537	0	92.0	90.1	90.7
ORF5	13762-14364	603	3	89.9	90.0	88.6
ORF6	14349-14873	525	3	93.7	92.8	93.3
ORF7	14863-15234	372	2	91.9	93.0	93.2
3'-UTR	15235-15385	151	2	91.4	88.7	91.5

Nucleotide comparison with VR-2332 demonstrated individual regions of NC16845 varied from 85.5% to 93.0% but the region corresponding to the ORF1a had the most variability primarily within the putative nsp2 region, similar to what has been previously reported with MN184 isolates (data not shown). In addition, NC16845 ORF1a contained high nucleotide variability compared to the ORF1a of JA142 and MN184 as well.

Nucleotide degeneracy was demonstrated in the ORF1a, ORF5, ORF6 and ORF7 regions of NC16845. In addition, the 5' and 3' UTRs also contained minimal degenerate bases in spite of their typical sequence conservation. The majority of the degenerate bases were located in the ORF1a region. This region of the PRRSV genome contains the putative nsp2 and nsp1β regions that have shown either nucleotide degeneracy or deletions in the genome in other virulent PRRSV. Collectively, the presence of nucleotide degeneracy suggests that more than one virus was present in the sample. In addition, the ORF5 region also contained moderate dissimilarity compared to JA142, VR-2332 and the MN184 (89.9%, 90.0%, and 88.6%, respectively). Interestingly, only the ORF1a showed a difference in nucleotide length. A discontinuous deletion of approximately 26 nucleotides was identified in this region. Comparison of ORFs2-7 to JA142 and VR-2332 did not reveal any differences in nucleotide length. Prepresentation of the NC16845 ORF5 nucleotide and amino acid sequence is represented in Fig. 1.

Fig. 1. NC16845 ORF5 envelope glycoprotein gene.

NC16845 ORF5 nucleotide bases

ATGTTCAAGTATGTTGGGGAAATGCTTGACCGCGGGCTTTTGCTCGCGATTGCTTTCTTTGTGGTGTATCGTGCC
GTTCTGGTTTGCTGTGCTCGTCGACGCCAACAGCACCAGCAGCTCTTATTCTCAGTTGATTTATAATTTGACGCT
ATGCGAGCTGAATGGCACAGACTGGCTGGATAAAAAATTCGATTGGGCAGTGGAGACTTTTGTCATCTTTCCC
GTTTTAACTCACATTGTCTCCTATGGTGCTCTTACTACCAGCCATTTCCTTGACACAGTTGGTCTGGCCACTGTG
TCCACCGCCGGATTTTATCATGGGCGGTATGTCTTAAGTAGCATCTACGCGGTCTGTGCCTTGGCTGCGTTGATT
TGTTTTGTCATTAGGTTTGCAAAGAATTGCATGTCCTGGCGCTACTCTTGTACCAGACATACCAACTTCCTCCTA
GACACTAAAGGCAGACTCTACCGCTGGCGGTCGCCCGTTATCATAGAGAAAGGGGGCAAGGTTGAGGTCGAC
GGCCATCTGATCGACCTCAAAAGAGTTGTGCTTGATGGTTCCGTGGCAACGCCTTTAACCAGAGTTTCAGCGGA
GCAATGGGG

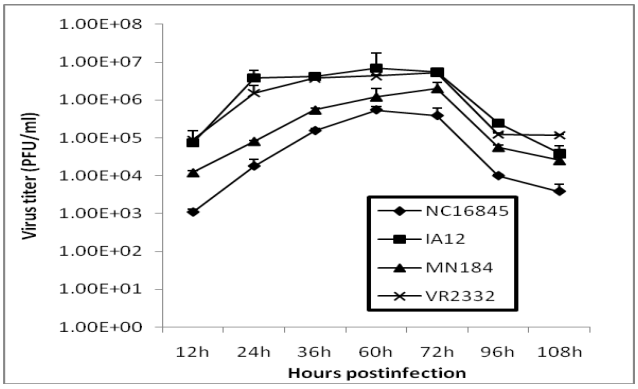
NC16845 ORF5 amino acids

MLGKCLTAGFCSRLLSLWCIVPFWFAVLVDANSTSSSYSQLIYNLTLCELNGTDWLDKKFDWAVETFVIFPVLTHIV
SYGALTTSHFLDTVGLATVSTAGFYHGRYVLSSIYAVCALAALICFVIRFAKNCMSWRYSCTRHTNFLLDTKGRLYR
WRSPVIIEKGGKVEVDGHLIDLKRVVLDGSVATPLTRVSAEQWGRP

Growth properties

Growth Curves: To evaluate the ability of NC16845 to grow *in vitro* compared to the IA12, VR-2332 and MN184, T25 flasks with confluent MARC-145 cells were infected with each virus in parallel at an MOI of 0.01. Supernatant samples were harvested at different time points and analyzed for viral yield by plaque titration. NC16845 had a rate of growth that was slower than the other three viruses (Fig. 2). However, the NC16845 growth rate was similar to MN184 compared to the other PRRSV used in the assay. At 60 hrs post-infection, NC16845 and VR2385 grew to a peak titer of $5.4x10^5$ and $7.0x10^6$ PFU/ml, respectively. At 72 hrs, MN184 and VR-2332 grew to peak titers of $2.0x10^6$ and $5.3x10^6$ PFU/ml, respectively. Overall, NC16845 titer was 4-13-fold less compared to MN184, VR-2332 and VR2385.

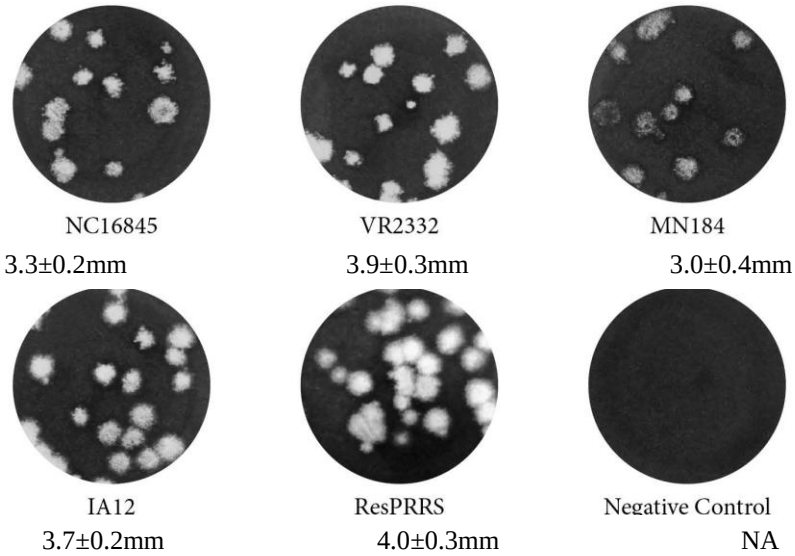
Fig. 2. One step growth curves expressed as mean plaque forming unites per ml from two independent experiments with error bars representing standard error of the mean.



Plaque Analysis: The plaque sizes were used to reflect the replication kinetics of each of the viruses used in the analysis including NC16845, VR-2332, MN-184 and VR2385. The NC16845 resulted in similar plaque sizes to the IA12. However, these two plaque sizes were only slightly larger than the MN-184, which has been previously shown to have a smaller, diffuse plaque size than other prototype PRRSV that appear larger and more definitive (Wang *et al.*, 2008). The plaque sizes of NC16845, MN-184 and VR2385 were smaller compared to those with the VR-2332 isolate. These results are also similar to previous reports using MN-184 and VR-2332 (Wang *et al.*, 2008). Although not a direct measure of viral virulence, plaque size appears to be indirectly proportional to *in vivo* virulence characteristics. Larger plaque sizes, exemplified in the VR-2332

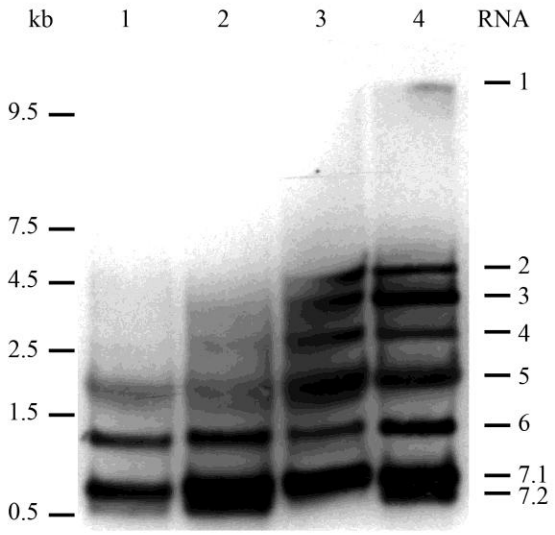
isolate, have been shown to be less pathogenic in pigs whereas the opposite is true for isolates known to be more pathogenic (MN-184) (Wang *et al.*, 2008). NC16845 was isolated from a swine herd experiencing excessive morbidity and mortality. Consistent with the previous statement, the plaque size for the NC16845 is smaller than the VR-2332 plaque. In addition, the IA12 virus, although isolated in late 1990, appears to have a similar although slightly larger plaque size to the NC16845. Ingelvac PRRS© MLV (aka ResPRRS) was included for comparative purposes.

Fig. 3. Plaque phenotypes of all four viruses, Ingelvac PRRS© MLV and negative control stained with crystal violet.



Northern Blot Assay: To further examine the replication properties of NC16845 compared to the other viruses, a Northern blot assay was performed using a digitoxin-labeled probe complementary to 3' UTR (Yuan *et al.*, 2000). This is a new procedure that has not been utilized in our lab replacing the use of radioactive-labeled probes which have not been approved for use in our lab. The success of this assay was marginal as the probes detected the smaller size RNA, but did not detect larger RNA or the full-length genome of some of the viruses. There are features of the different viruses that are apparent in the blots shown in Fig. 4. Analysis of RNA that was detected demonstrated that NC16845 and MN184 had similar patterns of subgenomic RNA and conversely, VR-2332 and VR2385 had similar patterns of subgenomic RNA primarily due to the presence of band 7-2. Interestingly, heteroclite species were not apparent in the gel depicted in figure 4 although the success of the hybridization does not indicate their absence, but perhaps the lack of detection.

Fig. 4. Northern blot hybridization of total RNAs isolated from MARC-145 cells infected with each virus at an m.o.i. of 0.01. RNA samples were hybridized with digitoxin-labeled probes complementary to PRRSV 3' UTR. mRNA numbers represent the PRRSV-specific mRNA species (mRNA represents the genome). RNA sizes were evaluated with an RNA marker (Invitrogen).



Lane 1: NC16845; Lane 2: IA12; Lane 3: MN184; Lane 4: VR-2332

Discussion

In this report, a novel PRRSV isolate, NC16845, was sequenced, characterized and compared to prototype PRRSV VR-2332 and additional isolates of comparable virulence for sequence analysis. NC16845 was isolated from a swine farm in North Carolina in 2006 from twelve-week-old pigs with a history of dyspnea, coughing and weight loss. The lungs were diffusely mottled tan in appearance, failed to collapse and contained interlobular edema. Due to the severity of clinical signs and excessive mortality, NC16845 was suspected to be a novel PRRSV of economic importance. Sequencing and *in vitro* growth analysis was thought to help elucidate unique features of the virus and provide information for a potential future vaccine.

Sequencing NC16845 revealed a nucleotide length of 15,385 bases comparable to the prototype VR-2332 and the VR2385 isolate which had the highest sequence homology to NC16845. The RFLP cut pattern is designated as 1-18-2, however, NC16845 is genetically distinct from other strains designated as RFLP 1-18-2, which have been recently isolated and are also known to cause severe disease in swine. The ORF1a region of the genome was the most variable compared to JA142, VR-2332 and MN184 (78.7 to 88.6% homology) consistent with previous characterization of additional virulent isolates with variations and deletions primarily in the ORF1a region of the genome (Han *et al.*, 2006). NC16845 also demonstrated variations within ORF5 that ranged between 88.6 to 90% homology with JA142, VR-2332 and MN184. ORF5 encodes a heterogeneous PRRSV structural protein and is often used for PRRSV diagnostic identification due to variations that exist between differing isolates (Kapur *et al.*, 1996). Interestingly, the ORF1a was the only region of the genome to vary in length compared to prototype VR-2332. Discontinuous deletions were noted in the putative nsp2 region of NC16845 adding to the variability that has also been reported in other isolates (Han *et al.*, 2006). NC16845 ORF1a was twenty-six nucleotides shorter compared to prototype VR-2332.

Nucleotide degeneracy was demonstrated in multiple trace files suggesting that more than one species of PRRSV was present in the sampled swine. Considering this isolate was derived from a field case of severe PRRSV, this is not unusual. Often, PRRSV consists of several individual species referred to as a swarm of related but distinct viral sequences, within infected animals. Interestingly, the majority of degenerate bases were present in the ORF1a as well as ORF5 with similar numbers of degenerate bases in ORF6 and 7 as well as the 5' and 3' UTR. The ORF6 and 7 genes code for the matrix and nucleocapsid protein that can contain moderate nucleotide variability as well.

Growth characteristics of NC16845 were compared to VR-2332, MN184 and an isolate used in an *in vivo* study, VR2385. VR2385 PRRSV is a virulent strain isolated in the 1990's and was used to represent a historical PRRSV for comparative purposes. Only the ORF2-7 regions of VR2385 have been sequenced to date. Due to this obstacle, sequence comparison was not conducted against VR2385. NC16845 appeared similar in growth characteristics to the MN184 virus although there were few moderate differences. Similar to MN184,

NC16845 appeared to replicate at a slower rate than the VR2385 and VR-2332 PRRSV. Consistent with previous suggestions, virulent isolates appear to replicate at lower rates in cell culture compared to less virulent strains. In addition, the VR2385 and VR-2332 isolates had similar rates of replication in cell culture which could indicate similar kinetics between these two strains. Studies are in progress to evaluate the virulence of NC16845 compared to VR2385 *in vivo*.

Although not a direct measure of viral virulence, plaque size appears to be indirectly proportional to *in vivo* virulence characteristics similar to the replication kinetics in cell culture. However, NC16845 had plaque sizes comparable to the VR2385 that were larger than the virulent MN184, but smaller than prototype VR-2332 which is less virulent *in vivo*. NC16845 plaque sizes were clear and distinct, lacking the diffuse appearance reported for MN184 isolates (Han *et al.*, 2006). Northern blot analysis also indicated differences between the NC16845 isolate and other PRRSV. Subgenomic RNA patterns were similar between NC16845 and MN184 compared to the patterns demonstrated by VR2385 and VR-2332. Interestingly, recently identified virulent PRRSV isolates appear to have fewer heteroclite species compared to historical isolates, although this feature was not apparent in Fig. 4. Further analysis is necessary to elucidate the heteroclite differences that may be present.

This genome analysis has increased our understanding of the immense nucleotide variation that exists in the field. The identification of increasing nucleotide variation in novel isolates as well as discontinuous deletions in the genome suggests that PRRSV continues to evolve to eliminate dispensable genomic regions and make the genome more compact (Han *et al.*, 2006). This variation could be due to increased exposure to different PRRS viruses, transport of swine over greater distances, swine management practices or simply due to the inherent properties of the virus. Consistent with previous reports, (Han *et al.*, 2006) sequence analysis of NC16845 supports the extreme nucleotide variability apparent in the nsp2 and ORF5 regions of the genome. Further monitoring of virulent PRRSV isolates is warranted to elucidate emerging virulence factors that could lead to the success of future elimination of this disease.

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