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Viral characteristics of plaque variants of porcine reproductive and respiratory syndrome virus

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Abstract : Plaque characteristics of porcine reproductive and respiratory syndrome (PRRS) virus isolates were examined using MARC-145 line cells. The plaque morphology of PRRS virus isolates was variable in size and heterogenic in population. Upon serial passages of the PRRS virus isolates on MARC-145 cells, heterogeneity was maintained but numbers of the large plaque size virus were increased with certain isolates. A PRRS virus isolate with variable plaque sizes was subcloned into 2 populations : small plaque (H_S) and large plaque (H_L) viruses. Growth kinetics of the subclones were then determined in MARC-145 cells, and production of the structural polypeptides was analyzed by SDS-PAGE. In a comparison of the growth kinetics, the H_S virus showed higher infectivity titers during the first 48 hours but slower to reach the peak titier than H_L virus did. In a nucleotide sequence comparison, differences of 4 nucleotides in open reading frames 5-6 gene were found between H_S and H_L viruses. Both the H_S and H_L clones produced 5 polypeptide bands with molecular weights of 15, 19, 26, 36 and 42 kD. The 5 bands were detected at 48 hours postinoculation (PI) with antisera to H_L and another large plaque virus (W_L) and at 72 hours PI with H_S virus antiserum. The present results demonstrate differences of biologic and molecular characteristics between the two PRRS virus plaque clones.

Key words : PRRSV, plaque variants, growth kinetics, SDS-PAGE, ORF5-6.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus was first isolated in Europe[1] and North America[2,3] as the cause of a new disease in swine. The PRRS virus is now classified into the family *Arteriviridae* along with lactate dehydrogenase elevating virus, equine arteritis virus and

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simian hemorrhagic fever virus. They share structural, genomic, and biological properties within the family members[1,4-6]. Replication of PRRS virus showed a limited *in vitro* cell susceptibility, and viral growth is only achieved in porcine alveolar macrophage cultures and some clones of monkey kidney cell lines[1,7]. Three major polypeptides have been described for both North American and European PRRS virus strains : a nucleocapsid polypeptide (N ; 15kD), an unglycosylated transmembrane polypeptide (M ; 19kD), and glycosylated envelope polypeptides (E ; 24-26kD)[8,9]. Antigenic and molecular differences have been demonstrated between the U.S. and European PRRS virus isolates[10,11]. Pathogenic and antigenic variations were also reported among the U.S. isolates[9,12].

We have observed PRRS virus isolate from the field outbreak with various plaque sizes. Certain isolates were a mixture of virus population with various plaque sizes. At this time, *in vitro* biological properties of the plaque variants are not known. The objectives of this study were to examine plaque characteristics of different PRRS virus isolates, investigate growth kinetics, viral polypeptide synthesis in MARC-145 cell line and compare nucleotide sequences of the selected PRRS virus plaque variants.

Materials and Methods

Cell cultures and viruses : Cell culture used was a permissive clone (MARC-145) of MA-104 cell line[7]. The MARC-145 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 3% fetal bovine serum (FBS), 0.15% sodium bicarbonate and antibiotics. Thirty PRRS virus field isolates were used from a panel of the virus isolates for an examination of their plaque morphology. Twenty-six of the isolates were obtained from the Oxford Veterinary Laboratories. All of the isolates were made from the farms before a PRRS modified live virus vaccine was implemented and had been passaged no more than 5 times in MARC-145 cells. Two PRRS virus plaque variants (H_L and H_S) which were subclones of a field virus and a large plaque variant (W_L) were initially established in this laboratory, and these were utilized for the examination of growth kinetics, viral structural protein synthesis and nucleotide sequence analysis.

Examination of viral plaque morphology : The palque test was performed by a method described previously[13]. Briefly, confluent MARC-145 cell monolayers grown in petri dishes (60mm × 15mm) were inoculated with each virus, and the inoculum was removed after 1 hour incubation at 37℃. The culture was washed once with MEM, and 5ml of a liquid culture medium consisting of an equal volume of double strength MEM and 2.4% boiled agar, supplemented with 100μg diethylamino-etyl-(DEAE)-dextran/ml and 2% FBS. The plates were incubated for 5 days at 37℃ in a CO_2 incubator, and the cell monolayers were stained with 2ml of 1% crystal violet in 20% ethanol for 10 minutes. Plaque size variants were determined on a plate which forms at least 10 plaques. A large-size plaque was considered to be ≥3mm in diameter, a medium-size plaque 2-〈3mm in diameter and a small-size plaque 〈 2mm in diameter.

In order to determine morphological change of the plaque upon passages, 3 PRRS virus isolates (OVL-135, OVL-112 and OVL-99) were subjected to a serial passage in MARC-145 cells up to 30 times. Each virus was collected when cytopathic effects reached at 70~80% and kept at -70℃ until plaque assay. The plaque-size subpopulation was evaluated on the virus passages 1, 7, 13, 19, 25 and 30.

One-step growth curves : Growth kinetics of PRRS virus plaque variants between H_L and H_S were compared. The MARC-145 cells (approximately 3.0×10^5 cells per tissue culture tube) were allowed to form a monolayer for 24 hours. The 2 plaque variant viruses (2ml, 10^5 $TCID_{50}$/ml) were inoculated into the tubes. After 30 minutes virus adsorption, the monolayers were washed twice with MEM, and then fresh MEM containing 4% FBS was replaced. Two tubes were sampled at the known intervals, and infectivity of the virus pools was determined and calculated by the method of Reed and Muench[14].

RNA sequence analyses : Oligonucleotide primers were synthesized on an automated DNA synthesizer according to nucleotide sequence information from the PRRS virus VR-2332. Open reading frames (ORFs) 5-6 gene was amplified by reverse transcriptase polymerase chain reaction,

and cDNA sequencing was done by an automated DNA fragment analyzer (ABI 377). The procedures and analyses were carried out in the Advanced Genetic Analysis Center, University of Minnesota.

SDS-PAGE : In order to examine viral polypeptides synthesis at different times postinoculation (PI), 3 plaque variants of PRRS virus (H_L, H_S and W_L) were metabolically labeled with ^{35}S-methionine (TRAN^{35}S-LABEL, ICN Biomedicals) for 4 hours, and the cell lysates were cross-immunoprecipitated with polyclonal swine sera against PRRS virus plaque variants (H_L, H_S and W_L). Antisera used were from pregnant sows which were inoculated with each PRRS virus plaque variant and collected at 33-46 days PI in a previous experiment[15]. The sera showed serum neutralizing antibody titers between 1 : 2-1 : 8 and IFA titers 1 : 256-1 : 1,024.

For the cross immunoprecipitation, MARC-145 cells were allowed to form monolayer in 75cm^2 of tissue culture flask for 24 hours. The viruses were inoculated with a titer of 10^6 $TCID_{50}$ per flask. The flasks were incubated for 24, 48 and 72 hours, respectively. The infected cells were labeled with 100μCi of S^{35}-methionine. Hot cell lysate preparations at the known intervals (1 ml) were reacted with different swine antisera (10μl), and then 100μl of a 10% protein A sepharose CL-4B (Pharmacia Biotech) was added. The prepared immunoprecipitates were resuspended with 100μl of 1x sample buffer and boiled for 3 minutes. The loading samples were subjected to SDS-PAGE[16] at 35-40mA/gel for 2.5 hours and analyzed by autoradiography. The gels were stained with Coomasie brilliant blue, destained, pretreated with 1M sodium salicylate for 20-30 minutes, dried and exposed to X-ray film (X-OMAT, Kodak) at -70℃ for 2-4 days. The molecular weights of the viral polypeptides were calculated by comparison with a broad range molecular weight standards (BioRad Lab., CA).

Results

Plaque morphology of PRRS virus isolates : Plaque morphology of the 30 PRRS virus isolates were examined in MARC-145 cells (Table 1). Three of the 30 isolates showed a mixture of virus with various plaque sizes : large, medium

Table 1. Heterogeneity in plaque sizes of porcine reproductive and respiratory syndrome (PRRS) virus isolates

PRRS virus isolate[a]	No. of plaque (in diameter)			Virus titer[b] (-Log^{10} $TCID_{50}$/ml)
	≥3mm	2-〈3mm	〈2mm	
MN-W	1	13	2	4
MN-1b	7	6	9	4
OVL-220	5	9	20	1
OVL-73		6	11	4
OVL-135		9	14	4
OVL-214		3	19	4
OVL-112		4	15	3
OVL-132		5	10	3
OVL-186		2	17	3
MN-H		4	8	3
OVL-110		5	30	2
OVL-161		2	12	2
702-5			50	5
OVL-11			34	4
OVL-99			41	4
OVL-173			20	4
OVL-202			15	4
OVL-30			28	3
OVL-114			28	3
OVL-132-1			13	3
OVL-113			43	2
OVL-174			27	2
OVL-180			26	2
OVL-211			23	2
OVL-215			23	2
OVL-217			12	2
OVL-93			28	1
OVL-184			23	1
OVL-212			12	1
OVL-218			20	1

[a]All PRRS virus isolates were passaged 〈5 times on MARC-145 cells.
[b]The lowest virus dilutions which plaque sizes were determined.

Table 2. Subpopulation of different plaque size of porcine reproductive and respiratory syndrome (PRRS) virus isolates through serial passage in MARC-145 cells

PRRS virus	Plaque size	Level of serial passage					
		1	7	13	19	25	30
OVL-135	L	0[a]	0	0	9	8	11
	M	5	4	0	3	5	21
	S	12	49	19	17	7	0
	pfu	1.7×10^4	5.3×10^3	1.9×10^5	2.9×10^4	2.0×10^5	3.2×10^5
OVL-112	L	0	14	5	13	19	7
	M	0	8	3	4	8	26
	S	5	4	2	3	1	4
	pfu	5.0×10^2	2.6×10^4	1.0×10^5	2.0×10^5	2.8×10^5	3.7×10^5
OVL-99	L	0	0	2	0	0	0
	M	0	0	11	10	11	23
	S	13	12	13	12	19	16
	pfu	1.3×10^4	1.2×10^5	2.6×10^4	2.2×10^5	3.0×10^5	3.9×10^5

[a]No. of plaque.
L = ≥3mm (in diameter), M = 2-〈3mm, S = 〈2mm.
pfu = plaque forming unit (ml).

and small in diameter. Nine isolates of PRRS virus contained 2 different plaque variants: medium- and small-size plaques. The remaining 18 isolates showed only small-size plaques.

Serial passage of 3 PRRS virus isolates (OL-135, OVL-112 with medium- and small-size plaque, and OVL-99 with small-size plaque) in MARC-145 cells resulted in production of the subpopulations with large- or medium-size plaques (Table 2). The numbers of large-size plaque virus increased markedly from the passage 19 for OVL-135 and passage 7 for OVL-112, but largely medium-size plaque increased from the passage level 13 for OVL-99.

One-step growth kinetics of plaque variants of PRRS virus : Two plaque variants, H_L and H_S showed a similar growth kinetics in MARc-145 line cells. Progeny virus was detected from 18 hours PI, and the H_S virus showed higher infectivity titers during the first 48 hours but slower to reach the peak titer than H_L virus did. The highest titers of the H_L was $10^{6.5}$ $TCID_{50}$/0.1ml at 72 hours PI, and that of the H_S virus was $10^{6.0}$ $TCID_{50}$/0.1ml at 96 hours PI (Fig 1). The infectivity decreased after the highest titers.

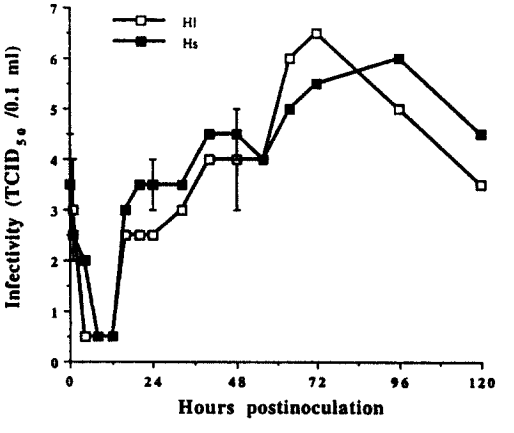

Fig 1. One-step growth curves of two plaque variants (H_L and H_S) of PRRS virus in MARC-145 cell.

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                    10         20         30         40         50
VR2332.SEQ      1 ATGTTTAAGT ATGTTGGAGA AATGCTTGAC CGCGGGCTGT TGCTCGCGAT     50
HL.SEQ          1 ********** *******G** ********** ********** *******A**     50
HS.SEQ          1 ********** *******G** ********** ********** *******A**     50

                    60         70         80         90        100
VR2332.SEQ     51 TGCTTTCTTT GTGGTGTATC GTGCCGTTCT GTTTTGCTGT GCTCGCCAAC    100
HL.SEQ         51 ******T*** ********** *******CT* ******T**C *****T**G*    100
HS.SEQ         51 ******T*** ********** *******CT* ******T**C *****T**G*    100

                   110        120        130        140        150
VR2332.SEQ    101 GCCAGCAACG ACAGCAGCTC CCATCTACAG CTGATTTACA ACTTGACGCT    150
HL.SEQ        101 ****A**G*A ********** AA**T***** ********** **********    150
HS.SEQ        101 ****A**G*A ********** AA**T***** ********** **********    150

                   160        170        180        190        200
VR2332.SEQ    151 ATGTGAGCTG AATGGCACAG ATTGGCTAGC TAACAAATTT GATTCGGCAG    200
HL.SEQ        151 ********** ********** ********** ***T****** **C*******    200
HS.SEQ        151 ********** ********** ********** ***T****** **C*******    200

                   210        220        230        240        250
VR2332.SEQ    201 TGGAGAGTTT TGTCATCTTT CCCGTTTTGA CTCACATTGT CTCCTATCGT    250
HL.SEQ        201 *****T**** ******T*** **T**G**** ********** ***T******    250
HS.SEQ        201 *****T**** ******T*** **T**G**** ********** ***T******    250

                   260        270        280        290        300
VR2332.SEQ    251 GCCCTCACTA CCAGCCATTT CCTTGACACA GTCGCTTTAG TCACTGTGTC    300
HL.SEQ        251 ********** *T******** ********** ****G*C*G* **********    300
HS.SEQ        251 ********** *T******** ********** ****G*C*G* **********    300

                   310        320        330        340        350
VR2332.SEQ    301 TACCGCCGGG TTTGTTCACG GGCGGTATGT CCTAAGTAGC ATCTACGCGG    350
HL.SEQ        301 *********A ********** ********** T**G****** **********    350
HS.SEQ        301 *********A ********** ********** T**G****** **********    350

                   360        370        380        390        400
VR2332.SEQ    351 TCTGTGCCCT GGCTGCGTTG ACTTGCTTCG TCATTAGGTT TGCAAAGAAT    400
HL.SEQ        351 ********** ********** *T******** *T******C* ***G******    400
HS.SEQ        351 ********** ********** *T******** *T******C* ***G******    400

                   410        420        430        440        450
VR2332.SEQ    401 TGCATGTCCT GGCGCTACGC GTGTACCAGA TATACCAACT TTCTTCTGGA    450
HL.SEQ        401 ********** ********T* A********* ********** **********    450
HS.SEQ        401 ********** ********T* A********* ********** **********    450

                   460        470        480        490        500
VR2332.SEQ    451 CACTAAGGGC AGACTCTATC GTTGGCGGTC GCCTGTCATC ATAGAGAAAA    500
HL.SEQ        451 ********** ********** ********** ********** **********    500
HS.SEQ        451 ********** ********** ********** ********** **********    500

                   510        520        530        540        550
VR2332.SEQ    501 GGGGCAAAGT TGAGGTCGAA GGTCATCTGA TCGACCTCAA AAGAGTTGTG    550
HL.SEQ        501 ********** ********** *****A**** ********** **********    550
HS.SEQ        501 ********** ********** *****A**** ********** **********    550

                   560        570        580        590        600
VR2332.SEQ    551 CTTGATGGTT CCGTGGCAAC CCCTATAACC AGAGTTTCAG CGGAACAATG    600
HL.SEQ        551 ********** ***C****** ****G***** ********** **********    600
HS.SEQ        551 ********** ***C****** ****G***** ********** **********    600

                   610        620        630        640        650
VR2332.SEQ    601 GCGTCGTCCT TAGATGACTT CTGTCATGAT AGCACGGCTC CACAAAAGGT    650
HL.SEQ        601 ********** ********** ********** ********** **********    650
HS.SEQ        601 ********** ********** ********** ********** **********    650

                   660        670        680        690        700
VR2332.SEQ    651 GCTTTTGGCG TTTTCTATTA CCTACACGCC AGTGATGATA TATGCCCTAA    700
HL.SEQ        651 ***C****** ********** ********** ********** **********    700
HS.SEQ        651 ***C****** ********** ********** ********** **********    700

                   710        720        730        740        750
VR2332.SEQ    701 AGGTGAGTCG CGGCCGACTG CTAGGGCTTC TGCACCTTTT GATCTTCCTG    750
HL.SEQ        701 ****A***** ********** ********** ********** **********    750
HS.SEQ        701 ****A***** ********** ********** ********** **********    750

                   760        770        780        790        800
VR2332.SEQ    751 AATTGTGCTT TCACCTTCGG GTACATGACT TTCGCGCACT TTCAGAGTAC    800
HL.SEQ        751 ********** ********** *********A ****T***** **********    800
HS.SEQ        751 ********** ********** *********A ****T***** **********    800

                   810        820        830        840        850
VR2332.SEQ    801 AAATAAGGTC GCGCTCACTA TGGGAGCAGT AGTTGCACTC CTTTGGGGGG    850
HL.SEQ        801 ********** ********** ********** ********** **********    850
HS.SEQ        801 ********** ********** ********** ********** **********    850

                   860        870        880        890        900
VR2332.SEQ    851 TGTACTCAGC CATAGAAACC TGGAAATTCA TCACCTCCAG ATGCCGTTTG    900
HL.SEQ        851 ********** ********** ********** ********** **********    900
HS.SEQ        851 ********** ********** ********** ********** **********    900

                   910        920        930        940        950
VR2332.SEQ    901 TGCTTGCTAG GCCGCAAGTA CATTCTGGCC CCTGCCCACC ACGTTGAAAG    950
HL.SEQ        901 ********** ********** ********** ********** **********    950
HS.SEQ        901 ********** ********** ********** ********** **********    950

                   960        970        980        990       1000
VR2332.SEQ    951 TGCCGCAGGC TTTCATCCCA TTGCGGCAAA TGATAACCAC GCATTTGTCC   1000
HL.SEQ        951 ********** ********** ********** ********** **********   1000
HS.SEQ        951 *********T *C******** ********** *T******** **********   1000
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                        1010        1020        1030        1040        1050
VR2332.SEQ      1001 TCCGGCGTCC CGGCTCCACT ACGGTCAACG GCACATTGGT GCCCGGGTTA 1050
HL.SEQ          1001 ********** ********** ********** ********** ********** 1050
HS.SEQ          1001 ********** ********** ********** ********** ********** 1050

                        1060        1070        1080        1090        1100
VR2332.SEQ      1051 AAAAGCCTCG TGTTGGGTGG CAGAAAAGCT GTTAAACAGG GAGTGGTAAA 1100
HL.SEQ          1051 ********** ********** ********** ********** ********** 1100
HS.SEQ          1051 ********** ***G****** ********** ********** ********** 1100

                        1110        1120        1130        1140        1150
VR2332.SEQ      1101 CCTTGTCAAA TATGCCAAAT AA..... ..........  .......... 1150
HL.SEQ          1101 ******T*** ********** **..... ..........  .......... 1150
HS.SEQ          1101 ******T*** ********** **..... ..........  .......... 1150
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Fig 2. Nucleotide sequence comparison of two PRRS virus plaque variants (H_L and H_S). The ATCC VR-2332 sequences used in this alignment are based on the sequence data presented by Murtaugh *et al*[17].

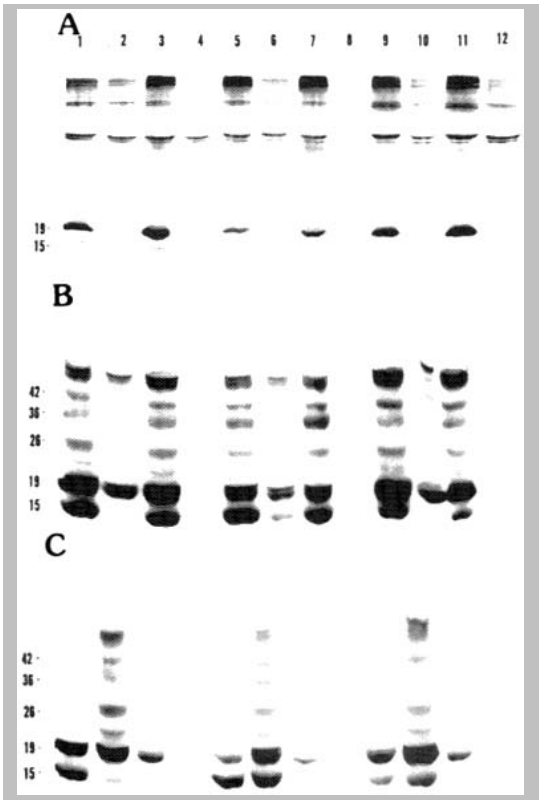

Fig 3. Comparison of the distribution of PRRS virus polypeptides in cell lysates.

H_L-(lane 1-4), H_S-(lane 5-8) and W_L-(lane 9-12) virus-infected cell lysates were immunoprecipitated with anti-H_L virus, anti-H_S virus, anti-W_L virus and negative control sow sera, respectively. The molecular weight of the virus proteins were calculated on the basis of the migration pattern of molecular weight standards included in each gel. Numbers in the left represent kilodaltons. A; 24 hours postincubation (PI), B; 48 hours PI, C; 72 hours PI.

Sequencing of ORFs 5-6 genome : A comparative sequence analysis showed a minor degree of difference between small (H_S) and large (H_L) plaque variants. The identity was 100% in ORF 5, and 99.3% of nucleotides and 98.3% of amino acids in OFR 6. Only 4 point mutations were observed in nucleotide 363 (C→T; Gly→Gly), 365 (T→C; Phe→Ser), 385 (G→T; Asp→Tyr) and 467 (T→G; Leu→Tyr) in genome encoding ORF 6 (Fig 2). However, there were some degree of differences between VR-2332 and the plaque variants. the nucleotide identity was 93.6% in ORF 5, and 99.0% between VR-2332 and the large plaque variant in ORF6 and 98.3% between VR-2332 and the small plaque variant in ORF 6.

Viral polypeptides synthesis of PRRS virus plaque variants : Cell lysates of 3 PRRS virus plaque variants (H_L, H_S and W_L) were cross-immunoprecipitated with 3 antisera to each plaque variant, and viral polypeptides synthesis was examined at 24, 48 and 72 hours PI, respectively. Two polypeptides with molecular sizes of 15 and 19kD were detected at 24 hours PI, but the amounts were markedly less against H_S antiserum (Fig 3A). At 48 hours PI, viral polypeptides were more obvious and additional bands with H_L and W_L virus antisera were detected at approximate molecular sizes of 26, 36 and 42kD polypeptides (Fig 3B). With H_S virus antiserum, the additional 3 bands were not clear at 48 hours PI, but obvious at 72 hours PI. At 72 hours PI, only 1 or 2 lower bands were detected with H_L and W_L virus antisera, while all of the 5 bands were detected with H_S virus antiserum (Fig 3C).

Discussion

Variable plaque sizes were evident when PRRS virus

field isolates were examined on MARC-145 cells. However, the majority (18 of 30) of the isolates were small plaque size with a homogeneous subpopulation. The initial isolation of each virus in the study was made using porcine alveolar macrophage or MARC-145 cell cultures. Continuous passages of the isolates in MARC-145 cells resulted in increased numbers of larger sized plaque subpopulations. This could be due to virus adaptation in the cells, while there may be a possibility of mutation or selection of the virus in the cells. Stability of the palque size of each variant might be analyzed because of the reason of decreasing the palque size at 30th passage on OVL-112. Therefore, determination of the plaque size evaluation for PRRS virus isolates should be examined at a low passage level. At this time, it is impossible to correlate any relationship between the plaque size and viral properties.

The results of nucleotide sequence analysis between H_L and H_S viruses were unexpected. Only 4 point mutations of the nucleotides and change of 3 amino acids in ORF 6 were observed. However, the results between VR-2332 ant the PRRS virus plaque variants support previous findings; ORFs 5, 3 and 7 seem to be the most variable[18], whereas ORF 6 is the most conserved[19]. Genetic distance between the 10 midwestern US isolates ranged from 2.5-7.9%[19]. Nucleotide sequence information of ORFs 2-4 and 7 is not available at this time. The significance of the change in ORF 6 is not known. It is necessary to define significant gene structure correlated to the palque size by comparing full genome sequence and several plaque variants. Although a full sequence information is required, the changes of ORF 6 may be significant for determining pathogenic differences between PRRS virus plaque variants H_L and H_S viruses.

In a cross-immunoprecipitation assay using the virus-infected cell lysates and antisera in sows raised against each virus, five specific viral polypeptides with molecular weights of 15, 19, 26, 36 and 42kD were demonstrated. Three major polypeptides of 15, 19 and 24-26kD were reported in the cell lysates of the North American strains of PRRS virus by immunoblotting with polyclonal antiserum or monoclonal antibodies[8,9]. In this study, we observed two additional polypeptides of molecular weights of 36 and 42kD. The origin of these polypeptides is not known at this time. Further characterization of the polypeptides would be required. However, in recent immunoblotting studies using monoclonal antibodies, 30-40kD and 40-50kD polypeptides were reported[20]. Preliminary results with monospecific antisera against bacterial fusion proteins suggested that the 30-40kD surface protein in encoded by ORF 4 of the PRRS virus genome and the 40-50kD protein by ORF 3. In other studies, the 45kD polypeptide was determined as the product of ORF 3[21]. The 42kD polypeptide has been reported as a highly glycosylated form of 26kD[8,22]. The number of glycosylation sites in a protein may affect the molecular mass of the protein in the gel[22]. Molecular weights of the 36 and 42kD found in this study were slightly different from other reports. The differences could be due to the method for measuring the molecular weight. Western blot method instead of radioimmunoprecipitation was used in most other studies[9,23].

This study supports previous reports on the synthesis of 3 viral specific polypeptides. Synthesis of viral polypeptides in infected cells with an American strain (ATCC VR-2332) was detected as early as 20 hours PI. The first polypeptides detected at 20 hours showed a molecular weight of 19kD. The polypeptide with a molecular weight of 15kD was detected only afte 30 hours PI. The three major polypeptides were optimally detected at 42 to 48 hours PI[24]. In this study, major differences in detection of the polypeptides were observed between the use of antisera and time at examination. Detection of the 5 bands was possible at 48 hours PI with H_L or W_L virus antisera, or 72 hours with H_S virus antiserum. It seems that there was no difference between the antisera of H_L and W_L viruses but marked difference between the antisera of H_L and H_S viruses.

Marked pathogenic difference between the 2 viruses was demonstrated[13]. Further investigation on the relation between the pathogenicity and the sequence differences along with amount or speed of the polypeptide synthesis would be required.

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