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Sequence analysis of ORF4 gene of porcine reproductive and respiratory syndrome virus (PRRSV) Korean isolate CNV-1

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Abstract : In this study PRRSV was isolated from serum of an infected pig and designated as CNV-1, ORF4 gene was sequenced, and the nucleotide sequence, deduced amino acid sequence and the amino acid sequence of the neutralizing domain was compared with other PRRSV strains.

ORF4 gene of the Korean isolate PRRSV CNV-1 was shown to be 537bp in length, which is the same as US strain ISU55 but 21bp longer than another US strain MN1b, and 15bp shorter than European strain LV. The homologies of the nucleotide sequences between the Korean isolate CNV-1 and the US strains ISU55, MN1b and European strain LV were 91.8%, 88.1%, 67.6%, respectively, and the homologies of the deduced amino acid sequences were 94.4%, 84.4%, 68. 5%, respectively. The neutralizing domain of the CNV-1 was shown to be 36 amino acids in length which is the same as ISU55, MN1b, but 4 amino acids shorter than that of the neutralizing domain reported in LV. The homologies of the amino acid sequences of the neutralizing domain between the Korean isolate CNV-1 and the US strains ISU55, MN1b and European strain LV were 92.5%, 85%, 57.5%, respectively.

The molecular characteristics of ORF4 gene of the Korean isolate PRRSV CNV-1 shown in this study suggests that the CNV-1 is genetically closer to the US strains. Also the wide variation of the neutralizing domain between the CNV-1 and LV suggests that there is substantial immunogenic variation between the two strains.

Key words : PRRSV ORF4 gene, genetic variation, neutralizing domain.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) has emerged as an important disease of swine throughout the world and is characterized by severe reproductive failure in sows and respiratory symptoms in all ages[1-6].

The PRRS virus is classified as a member of the genus *Arterivirus*, family *Arteriviridae* of which equine arteritis virus (EAV), lactase dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) are also members[7-11]. The genome of the virus, a positive-strand RNA, is 15kb in length and contains 8 open reading frames that encode viral proteins. ORFs 1a and 1b encode the viral RNA polymerase, and ORFs 2, 3 and 4 encode structural proteins of the virion[12]. ORF5 which encodes a 25-kDa *N*-glycosylated envelope glycoprotein is the most variable and ORF6 encoding the 18-kDa non *N*-glycosylated protein is the most conserved[13]. ORF7 encodes the 15kDa nucleocapsid protein. Recently, a neutralization domain in the protein encoded by ORF4 of Lelystad virus(LV) was identified and thus the region is considered to be antigenically involved[9]. Strain variation is a characteristics of this group of viruses. There are substantial antigenic and molecular variations among the PRRS virus strains[14-18]. In a comparison of 24 field sera and 7 viral isolates from Europe and US, European and US isolates were found to be immunologically distinct with no common antigens[16]. Also in a comparison of nucleotide sequence of a 3266bp region encompassing ORFs 2 through 7 of PRRS virus, genetic distances between US isolates were 5.8%$\pm$0.2% whereas the Lelystad strain from Europe was, on average, 34.8% divergent from US strains[15]. Although there is wide immunogenic and genetic variation between the US and European PRRSV isolates, little is known about the relationship between Korean strains, and US and European strains. This study reports the isolation of PRRSV Korean isolate CNV-1 and its molecular characteristics of ORF4 gene. The ORF4 gene was chosen for analysis for it's possible involvement in immunogenicity. The nucleotide and deduced amino acid sequences were compared with those of the US strains ISU55, MN1b and the European strain LV. Also the amino acid sequence of a reported neutralization domain identified in LV was compared with its corresponding sequences in the Korean isolate and the US strains.

Materials and Methods

Cell culture, virus isolation and identification : Isolation of the PRRS virus was attempted from serum, collected from a pig farm with symptoms of PRRS. The procedure for the virus isolation and identification have been previously described in detal[1,3,19,20]. 200μl of 10 fold diluted serum sample was cenrifuged and inoculated onto permissive cell line MARC-145, maintained in Eagle's minimum essential media. After incubation at 37℃ for 1 hour to allow virus adsorption, 0.8ml of MEM was added. Cytopathic effects (CPE) specific for PRRS virus were observed daily. IFA test was performed to confirm the isolate as being a PRRS virus.

RT-PCR : Viral RNA was prepared from virus culture media as previously described[21]. 200μl of the virus culture supernant was mixed with 2μl of RNasin (40units/ml) and 66μl of 10% Sodiumdodecyl sulfate (SDS). The mixture was incubated for 5 minutes at 50℃ and the solution was extracted with equal volume of phenol : chloroform. Viral RNA was precipitated in ethanol and pelleted by centrifugation at 12,000g for 20 minutes. The oligonucleotide primers used for reverse transcription and polymerse chain reaction (RT-PCR) was TTGGATCCATGGCTGCGGCCACTCTT(forward primer) and TTGAATTCTCATATTGCCAAGAGAAT (reverse primer). The primers were synthesized according to nucleotide sequence information from LV (EMBL/Genbank Data Libraries Accession No. M96262). The *Bam* HI and *Eco*RI restriction sites were incorporated at the 5' end of the primers to facilitate cloning. RT-PCR was performed according to the method reported previously[21]. The RT mixture was incubated at 37℃ for 2 hours in a DNA thermal cycler (Perkin-Elmer, USA) and PCR was performed at 40 cycles of denaturation at 94℃ for 1 minute, annealing at 45℃ for 2 minutes, and polymerization at 72℃ for 3 minutes. Polymerization step was extended to 7 minutes for the last cycle.

Cloning : The PCR product of ORF4 gene and plasmid vector pTZ18R was treated with *Eco*RI and *Bam*HI and then purified using Geneclean II kit (Bio 101, USA). DNA ligation kit (Novagen, USA) was used to ligate the PCR product into plasmid vector pTZ18R at the multicloning site. The resulting recombinant plasmid was designated pTZ18R-ORF4. Dh5a (Gibco BRL, USA) competent cells were prepared by calcium chloride method and used for cloning of the pTZ18R-ORF4. The cells were inoculated on selective LB agar plates containing ampicillin and well isolated colonies were picked from the plates. The plasmid DNA was isolated by a modified alkaline lysis procedure. The insert DNA was confirmed by electrophoresis on 1.2% agarose gel.

Nucleotide sequencing : Cycle sequencing using dye labeled terminators was performed using dye terminator ready reaction mix (Perkin-Elmer, USA) according to the manufacturers instructions. Forward pUC/M13 (Promega, USA) primer was used for the determination of forward sequence and a reverse pUC/M13 primer (Promega, USA) for the complement sequence. Cycle sequencing was performed on a DNA thermal cycler at 25 cycles of denaturation at 96℃ for 30 seconds, annealing at 50℃ for 15 seconds, and polymerization at 60℃ for 4 minutes. Sequence data was collected on a ABI prism 310 genetic analyzer (Perkin-Elmer, USA) and the nucleotide sequences were analyzed with Collection, Analysis, and SeqEd (Perkin-Elmer, USA) software.

Results

Cell culture, virus isolation and identification : The cell monolayer inoculated with the serum sample showed CPE typical for PRRS virus. The virus isolate was identified as PRRS virus by IFA test using PRRS virus antibody positive swine sera and designated as CNV-1 (Fig 1).

RT-PCR : Genomic viral RNA of the Korean isolate CNV-1 was isolated. The cDNA of the ORF4 was synthesized by reverse transcription and amplified by PCR. An appropriate band of about 500bp was seen on 1.2% agarose gel stained with ethidium bromide (Fig 2).

Cloning of ORF4 cDNA : The PCR product was ligated into linearized pTZ18R plasmid vector. The recombinant

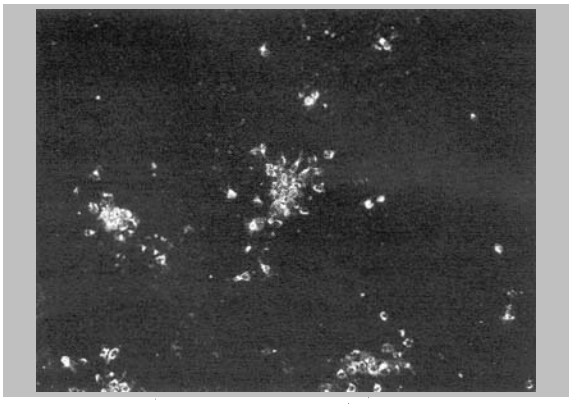

Fig 1. Indirect fluorescent antibody test of MARC-145 cells infected with CNV-1. Fluorecent color positive cells were observed in the infected cell monolayers.

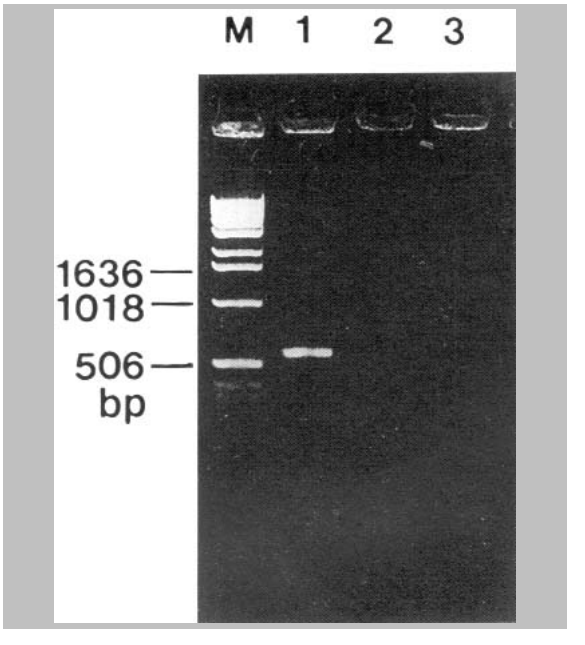

Fig 2. Agarose gel electrophoresis of ORF4 PCR product. Lane M : 1kb DNA ladder, lane 1 : PCR product.

plasmid DNA, pTZ18R-ORF4, was transformed into *E coli* DH5a and the presence of insert cDNA was confirmed by enzyme treatment and electrophoresis.

Nucleotide and amino acid sequence analysis : The nucleotide and deduced amino acid sequences of ORF4 of CNV-1 haven been determined and are shown in Fig 3. Nucleotide sequencing results showed the length of ORF4 of CNV-1 to be 537bp long, which was the same as ISU55 but

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ATGGCTGCGGCCACTCTTTTCCTCTTGGTTGGTTTTAAATGTTTCTTGGTTTCTCAGGCGTTTGCCTGCAAGCCGTGTTTCAGTTCG
MetAlaAlaAlaThrLeuPheLeuLeuValGlyPheLysCysPheLeuValSerGlnAlaPheAlaCysLysProCysPheSerSer

AGTCTTGCAGACATCAAGACCAACACCACCGCAGCAGCAGGCTTTGCTGTCCTCCAAGACATCAGCTGCCTTAGGTATGGCAACTCG
SerLeuAlaAspIleLysThrAsnThrThrAlaAlaAlaGlyPheAlaValLeuGlnAspIleSerCysLeuArgTyrGlyAsnSer

TCCTCTAAGGCGTTTCGCAAGATTCCTCAATGCCGCACGGCGATAGGGACACCCGTGTATATTACTGTCACAGCCAATGTAACTGAT
SerSerLysAlaPheArgLysIleProGlnCysArgThrAlaIleGlyThrProValTyrIleThrValThrAlaAsnValThrAsp

GAGAACTATCTACATTCTTCTGATCTTCTCATGCTTCCTTCTTGCCTTTTCTATGCTTCTGAGATGAGTGAAGAGGGATTCAAGGTG
GluAsnTyrLeuHisSerSerAspLeuLeuMetLeuProSerCysLeuPheTyrAlaSerGluMetSerGluGluGlyPheLysVal

GTATTTGGCAATGTGTCAGGCATCGTGGCTGTGTGTGTCAATTTTACCAGCTACGTCCAACATGTCAAGGAGTTCACTCAACGCTCC
ValPheGlyAsnValSerGlyIleValAlaValCysValAsnPheThrSerTyrValGlnHisValLysGluPheThrGlnArgSer

TTGATGGTCGATCATGTGCGGCTGCTCCATTTTATGACACCTGAAACTATGAGGTGGGCAACTGTTTTAGCCTGTCTTTTCGCCATT
LeuMetValAspHisValArgLeuLeuHisPheMetThrProGluThrMetArgTrpAlaThrValLeuAlaCysLeuPheAlaIle

CTCTTGGCAATATGA
LeuLeuAlaIle<U>
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Fig 3. Nucleotide sequence and deduced amino acid sequence of the ORF4 gene of Korean isolate CNV-1.

21bp longer than that of MN1b (516bp) and 15bp shorter than that of LV (552bp). The aligments of the nucleotide sequence and the deduced animo acid sequence are shown in Fig 4 and Fig 5, respectively. Comparison of the nucleotide and amino acid sequences showed the ORF4 of CNV-1 to be the closest to ISU55 strain with a homology of 91.8% for the nucleotide sequence and 94.4% for amino acid sequence. LV was the furthest with a homology of only 67.6% for the nucleotide sequence and 68.5% for amino acid sequence. The homology of nucleotide sequences and amino acid sequences between strains are summarized in Table 1. Neutralizing domain spanning amino acids 40 to 79 of LV ORF4 was further analyzed for the determination of possible antigenic differences between the strains. CNV-1, ISU 55, and MN1b were shown to have 4 amino acid deletions at the same positions when compared to LV. The homology was highest between CNV-1 and ISU55 at 92.5% and lowest between LV and MN1b at 50%. Homology of the neutralizing domain is summarized in Table 2.

Table 1. Nucleotide sequence and deduced amino acid sequence hemology between CNV-1 and ISU55, MN1b, and LV

	CNV-1/ISU55	CNV-1/MN1b	CNV-1/LV
Nucleotide sequence	537/537	537/516	537/552
Homology	91.8%	88.1%	67.6%
Amino acid sequence	178/178	178/171	178/183
Homology	94.4%	84.4%	68.5%

Table 2. Homology of the neutralizing domain among four strains of PRRSV

	CNV-1/LV	CNV-1/ISU55	CNV-1/MN1b	LV/ISU55	LV/MN1b	ISU55/MN1b
Neutralizing domain	36/40	36/36	36/36	40/36	40/36	36/36
Homology	57.5%	92.5%	85%	52.5%	50%	90%

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ISU55  ATGGCTGCGT CCCTTCTTTT CCTCTTGGTT GGTTTTAAAT GTCTCTTGGT TTCTCAGGCG
CNV-1  ---------G --AC------ ---------- ---------- --T------- ----------
MN1b   ---------G --AC------ ----A----- ---------- ---------- ----------
LV     ---------G --AC------- -T--C---C- ---GC-C--C A-A--A---- ----G-----

ISU55  TTCGCCTGCA AGCCATGTTT CAGTTCGA-G --TCTTGCAG ACATTAAGAC CAACACCACC
CNV-1  --T------- ----G----- ---------- ---------- ----C----- ----------
MN1b   ----D----- -AD-DDDDDD --DD-DDD-- --DD------ ---------- ----------
LV     --------T- ----C----- -DDD----C- CA---AT--- -T---G---- ------G---

ISU55  GCAGCGGCAG GCTTTGC-TG -TCCTCCAAG ACATCAGTTG CCTTAGGTAT CGCAACTCGG
CNV-1  -----A---- ---------- ---------- -------C-- ---------- G--------T
MN1b   ---------A ---------- ---------- ---------- -----,-C-- ----------
LV     --G--T--C- -D---D-A-- G----T--G- ------A--- DT--CC-D-C -T-D--GG--

ISU55  CCTCTG-AG- GC--G----- -TTTCG--CA -AAA-TCC-C TCAGTGTCGT -ACGGCGATA
CNV-1  -----A---- ---------- ---------- --G---T--- ---A--C--C ----------
MN1b   ---------- -----A---- -D-------- ---------- ------C--- GD--------
LV     T---A-C--C --AA-AGAAA A----CTT-G G---G--GT- C--A------ G-A-C--D-C

ISU55  GGGACACCCA TGTATATTAC TG-TCACAGC CAATGTAACC GATGAGAAT- -TA-TT-TGC
CNV-1  ---------G ---------- ---------- ---------T -------C- -----C--A-
MN1b   --------TG ---------- CA-------- ------G--A ---------- --------A-
LV     --T--T---C A---C--C-- D-A-A--G-- T--C--G--- --DDC----C A--C--G-A-

ISU55  ATTCCTCTGA CCTTCTCATG CTTTCTTCTT GCCTTTTCTA CGCTTCTGAG ATGAGTGAAA
CNV-1  ----T----- T--------- ---C------ ---------- T--------- ---------G
MN1b   ----T----- T--C------ --C------- ---------- T--------- ----------
LV     -DAD-G-G-- ---G--G--- ------G-G- ---------- ---C--A--A -----C--G-

ISU55  AGGGATTTAA AGTGGTATTT GGCAATGTGT CAGGCATCGT GGCTGTGTGC GTCAACTTTA
CNV-1  -------C-- G--------- ---------- ---------- ---------T -----T----
MN1b   --------G- G-----T--- ---------- ---------- ---------T -----T----
LV     -A--C--C-- ---CA-C--- --G-----C- -T---G-T-- TT---CT--T -----T--C-

ISU55  CCAGCT-ACG TCCAACATGT CAAGGAATTT ACCCAAC-GC -TCCT-TGGT AGTCGACCAT
CNV-1  ---------- ---------- ------G--C --T------- --------A- G-----T---
MN1b   ---------- ---------- --G---G--- -------T-- --------DD -D---DDDD-
LV     D---A-T-T- -GGCC----- G-CCC--CA- -----G-A-- A--A-C---- -A-T--T--C

ISU55  GTGCGGCTGC TCCATTTCAT GACACC-TGA GACCATGAGG TGGGCAACTG TTTTAGCCTG
CNV-1  ---------- -------T-- ---------- A--T------ ---------- ----------
MN1b   ---------- ---------- ---------- ---------- --------C- ----------
LV     A-T---T--- -G------C- ------A-CT -D-A------ -----T--AA CCA-T--T--

ISU55  TCTTTTT-GC CATTCTGTTG GCCATTTGA
CNV-1  ------C--- ------C--- --A--A---
MN1b   ---------- ------C--- --A--A---
LV     -D--G--C-- ------C--- --A--A---
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Fig 4. Multialignment of the ORF4 gene nucleotide sequences of CNV-1 (Korean isolate), ISU55 (US strain), MN1b (US strain), and LV (European strain). Deletions are represented by D.

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LV      MAAATLFFLA GAQHIMVSEA FACKPCFSTH LSDIETNTTA AAGFMVLQDI NCFRPHGVSA
CNV-1   --------L-V -FKCFL--Q- --------SS -A--K----- -----A----- S-L-dY-N-d
ISU55   ---SL--L-V -FKCLL--Q- --------SS -A--K----- -----A----- S-L-dYRN-d
MN1B    --------LMV -FKCLL--Q- --dddddAN- VA--K----- --S-A----- S-L-d-RN-d

LV      AQEKISFGKS SQCREAVGTP QYITITANVT DESYLYNADL LMLSACLFYA SEMSEKGFKV
CNV-1   dSS-dA-R-I P---T-I--- V---V----- --N--HSS-- ---PS----- -----E----
ISU55   dASEdA-R-I P---T-I--- M---V----- --N--HSS-- ----S----- ----------
MN1B    dASEdAIR-I P---A-I--- V--------- --N--HSS-- ----S----- --------E-

LV      IFGNVSGVVS AdCVNFTDYV AHVTQHTQQH HLVIDHIRLL HFLTPSAMRW ATTIACLFAI
CNV-1   V------I-d -V-----S-- Q--KEF-d-R S-MV--V--- --M--ET--- --VL------
ISU55   V------I-d -V-----S-- Q--KEF-d-R S--V--V--- --M--ET--- --VL------
MN1B    V------I-d -V-----S-- Q--REF-d-d d-LL-RV--- --M--ET--- --VL------

LV      LLAIJ
CNV-1   -----
ISU55   -----
MN1b    -----
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Fig 5. Multialignment of the deduced amino acid sequences of CNV-1 (Korean isolate), ISU55 (US strain), MN1b (US strain), and LV (European strain). Small letter d represents deleted amino acid sequences.

Discussion

In this study, the homologies of nucleotide and deduced amino acid sequences between the Korean isolate CNV-1 and US strains were shown to be higher than that of those between the Korean isolate and the European strain. This indicates the Korean isolate is genetically closer to the US strains than the European strain. Although the ORF4 is not thought to be uniquely involved in immunogenecity, it is the only known region to contain a neutralizing domain. Comparison of the amino acid sequence of neutralization domain between LV and CNV-1 showed much variation, with only 57.5% homology along the 40 amino acid length region. This was in accordance with a previous report that the neutralization domain was much more variable than other parts of the protein and that this domain might be susceptible to immuno selection[22]. The wide variation in the neutralizing region suggests that a substantial immunogenic difference exists between the CNV-1 and the LV. However, when the neutralizing domain of CNV-1 strain was compared with those of the US strains, the homology was considerably higher being 92.5% for ISU55 and 85% for MN1b. The higher homology seen in this neutralizing region between CNV-1 and the US strains suggest that the CNV-1 is also immunogenetically closer to the US strains than the European strains, although neutralizing regions in other structural proteins remain to be investigated. The data also suggest that the PRRS virus Korean isolate, might have originated from the United States rather than Europe.

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