

Cloning and Sequence Analysis of ORF5 Gene of Porcine Reproductive and Respiratory Syndrome Virus of the ZJ Strain

Baocheng Hao^{1,3} Dengyuan Niu^{1,2} Xiaoyong Xing¹ Haitao Xiang¹ Fengqin Wen¹

Xiaoping Fu¹ Jianping Liang^{1,3} Yonghao Hu^{1*}

Abstract

A pair of the special primers was designed and synthesized according to the ORF5 sequence of porcine reproductive and respiratory syndrome virus (PRRSV), which was published in GenBank. In 2009 a pig farm in Zhejiang province suspected some diseased and dead pigs displaying symptoms of porcine reproductive and respiratory syndrome and provided 12 samples (spleen, kidney or lymph nodes) for laboratory diagnosis. The samples were positive of porcine reproductive and respiratory syndrome by PCR. The ORF5 gene was amplified by RT-PCR and cloned into pMD-18T vector. Nucleotide sequence was determined by sequencing and compared with other ORF5 genes in GenBank. Then, the structure of ORF5 gene was analyzed. Results showed that the amplified fragment length was 603 bp, encoding 201 amino acids. Compared with the corresponding region of other PRRSV strains, the nucleotide sequence homology was 63.5% - 90.7% with other strains published in GenBank, amino acid sequence homology was 11.2% - 87.6% and the nucleotide sequence homology was 90.7% with AF494042 strain of American type. Therefore, we suggest that the isolates of ZJ strain belong to the American type. The ORF5 gene encoding the GP5 protein is the main structure protein and immune protein, involving the generation of neutralizing antibody. Thus, GP5 has good properties as new target protein of gene engineering vaccine and diagnosis kit. This study provides valuable reference for development of a vaccine based on GP5 protein.

Keywords: Cloning, ORF5, PRRSV, Sequence analysis

¹College of Veterinary Medicine, Gansu Agricultural University, Lanzhou 730070, PR China

²Institute of Animal sciences, Zhejiang academy of agricultural sciences, hangzhou 310021, PR China

³Key Laboratory of New Animal Drug Project, Gansu province/Key Laboratory of Veterinary Pharmaceuticals Discovery, Ministry of Agricultural /Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS, Lanzhou 730050, PR China

*Correspondence: yhh0817@126.com

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important swine pathogens which significantly affects swine industry because it is highly infectious and causes reproductive problems and respiratory distress. It also causes abortion, stillbirth, fetal mummies of pregnant sow. Now porcine reproductive and respiratory syndrome has become one of the main diseases in large scale pig farms. Recently, it has been shown that PRRSV replicates in the endometrium and placenta which is most probably a prerequisite for reproductive disorders (Karniychuk et al., 2011, 2012). The virus has a restricted tropism to sialoadhesin (Sn)-positive macrophages (Duan et al., 1998; Vanet al., 2003; Van et al., 2008).

PRRSV is divided into two distinct genotypes at present: the European type (type I) and the North American type (type II) (Meulenberg et al., 1993; Nelsen et al., 1999). The two genotypes are represented respectively by the Lelystad virus (LV) and the prototypes VR-2332 and share only about 60% identity at the nucleotide level (Suarez et al., 1996). There are considerable genetic variations among field viruses of the same genotype in different geographical locations and the coexistence of both genotypes has been increasingly done evidently in several countries, including Korea, Thailand and China (Kim et al., 2010; Lee et al., 2010; Tun, et al., 2011; Cao et al., 2012).

ORF5 of PRRSV genome is one of the most variable regions. The ORF5 gene of PRRSV encodes a glycosylated membrane protein (GP5 protein). GP5 protein contains 4 N-glycosylation sites, with 31 amino acids of a signal peptide and three transmembrane regions, respectively in the 62 - 83, 90 - 106, 113 - 130 amino acid (Liu et al., 2001). GP5 is one of the important immunogenic proteins for PRRSV which is militated against by an association with virus neutralization. The N-terminal ectodomain of the protein has the mapping point of the major neutralizing epitope of GP5 (Ostrowski et al., 2002; Plagemann, 2004). In addition, through heterodimers, GP5 associated with the M protein enables it to play important roles in viral attachment and macrophage internalization (Delputte et al., 2002). Genetic variation of GP5 has been reported in several studies from different regions and countries (Yin et al., 2009; Shang et al., 2012; Shi et al., 2013; Zhang et al., 2013).

Since 2006, huge economic losses have been caused by the emergence of highly pathogenic PRRSV in the swine industry of China. This study aimed to sequence and analyzes ORF5 of PRRSV isolates obtained from Zhejiang province in 2009 by Molecular cloning and sequence analysis of ORF5 of PRRSV. This study provides valuable reference for development of a vaccine based on GP5 protein in China.

Materials and Methods

Sample: Affected tissues (spleen, kidney and lymph nodes) were collected from clinical PRRSV suspected pigs. An appropriate amount of sterile saline was added with grinding. Supernatant obtained by centrifugation was added to double anti-penicillin and streptomycin (1000 IU/ml) and placed at 4 °C for 4-5 h. Monolayer cultured Marc-145 cells were inoculated and cultured for 72 h. Then, cell fluid of blind spread cells that showed obvious CPE was collected. Virus was released by the ruptured cells after freezing and thawing the cell fluid three times. Trizol was used to extract virus. The extracted virus was stored at -70 °C.

RNA extraction and RT-PCR: Total RNA was extracted by using TRizol reagent (Invitrogen Co., USA) according to the manufacturer's instructions and then used for synthesis of cDNA with random hexamers (Fermentas, USA).

The sense primer was 5'-ggtcattggtgttggggaatgcttgacc-3' and the reverse primer was 5'-taagaattctaatgatgatgatgatgatggagacgacccattgttc-3'. These specific primer pairs were used to amplify 621 bp amplicons encoding the complete ORF5. By using a one-step RNA PCR kit (Takara Co., Dalian, China), the RT-PCR was carried out. The cycle conditions were 42 °C for 60 min and 70 °C for 15 min for RT, 95 °C for 5 min (for hot start) and then 30 cycles at 94 °C for 60 sec, 50 °C for 60 sec and 72 °C for 1 min (for PCR amplification), and a final extension at 72 °C for 10 min. Eventually, by using 1% agarose gel electrophoresis under ultraviolet light, PCR products (2 µl) were visualized.

Cloning, nucleotide sequencing, and identifying: According to the instructions of manufacturer, MiniBEST Agrose Gel DNA Extraction Kit (TaKaRa Co., China) was used to recycle the PCR product. Then the PCR product was cloned into a PMD-18T vector (TaKaRa Co., China). Restriction endonuclease EcoR I and Hind III double digestion were used for identification. Correctly identified and reconstructed plasmid was sent to TaKaRa for sequencing.

Homology analysis of Nucleotide and amino acid sequences: The ORF5 gene sequence of ZJ strain was analyzed by Clustal X 2.0 program (DNASTAR Inc., USA), and compared with nucleotide and amino acid sequences of the 33 PRRSV strains published in Genbank. By using MEGA 5 software with the neighbor-joining method, a phylogenetic tree was constructed. Bootstrap values were calculated on 1000 replicates for alignment.

Results and Discussion

Using the RT-PCR method to amplify ORF5 gene from PRRSV cytotoxicity, the product was detected by 1% of agarose gel electrophoresis, the band

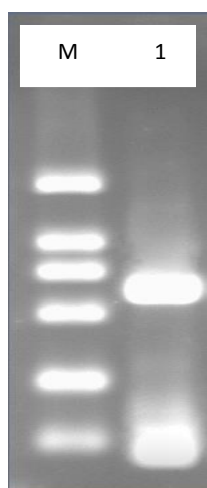


Figure 1 RT-PCR products of gene segment ORF5
M: 2000 bp DNA marker; 1: product of RT-PCR

of about 621bp, consistent with the expected fragment size (Fig 1).

For the determination and characteristic analysis of the nucleotide sequence of the ORF5 gene, the results showed that the full-length of ORF5 nucleotide sequence was 621 bp (Fig 2). The amplified ORF5 gene fragment contained the complete ORF5 sequence. The ORF5 fragment size was 603 bp, and encoded 201 amino acids (Fig 3) with 33344.24 Dalton molecular weight.

Nucleotide and amino acid sequences of the 33 PRRSV isolated strains were compared with those of AF159149, AF331831, AF494042, AY032626, AY366525, DQ473474, EF075945, EF112445, EF112446, EF112447, EF532817, EF535999, EF536002, EF635006, EU109503, EU106888, EU187484, EU200962, EU366151, EU807840, EU939312, FJ147205, FJ556871, FJ629369, FJ895329, FJ897565, GQ243425, GU067771, GU366151, KC862584,

KF991509, M96262, U87392. Sequence comparison showed that nucleotide homology was respectively 90.5%, 90.4%, 90.7%, 90.5%, 64.5%, 90.5%, 88.6%, 88.7%, 88.6%, 88.6%, 86.9%, 87.2%, 88.4%, 88.9%, 88.7%, 88.9%, 89.1%, 88.9%, 87.9%, 89.9%, 88.9%, 88.9%, 88.2%, 87.6%, 88.7%, 88.1%, 88.2%, 63.5%, 88.4%, 89.7%, 64.5%, 64.3% and 90.7%. This analysis showed that the 33 isolates shared 64.3% - 90.7% nucleotide identity (Fig 4). For the amino acid sequence comparison, amino acid homology was respectively 85.6%, 85.6%, 87.6%, 86.1%, 54.8%, 87.6%, 85.1%, 84.6%, 85.1%, 85.1%, 83.1%, 84.1%, 85.1%, 85.1%, 85.1%, 85.1%, 85.1%, 85.6%, 82.6%, 12.2%, 85.1%, 85.6%, 83.6%, 84.6%, 85.6%, 83.6%, 84.6%, 55.3%, 85.1%, 85.1%, 55.3%, 55.3%, 11.2% (Fig 5).

According to the nucleotide sequence of ORF5 and the phylogenetic tree drawn by Treeview software (Fig 6), results showed that the U87392 and AF159149 strains were close to the ZJ strain in genetic distance, the AY366525 and M96262 strains were farther away from the ZJ strain in genetic distance, and the M96262 strain was farthest from the ZJ strain in genetic distance.

The ORF5 gene of ZJ strain of PRRSV in the study was cloned and sequenced. It is inferred that the ZJ strain is the highly pathogenic PRRSV strain and the homology is higher than some domestic strains that are the highly pathogenic PRRSV strains. The nucleotide homology with the AF494042 strain was 90.7%. The AF494042 strain is a typical American strain. Therefore, it is inferred that the ZJ strain belongs to the American type.

The ORF5 gene encoding the GP5 protein is the main structure protein and immune protein, involving the generation of neutralizing antibody. Therefore, GP5 is suitable target protein for gene engineering vaccine and diagnosis kit.

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ATGTTGGGAAATGCTTGACCGGGGCTATTGCTCGTCATTGCTTTTTTTGTGGTGATCGTGCCGTCTTGTTGCGCTCGCCAGCGCCAACAGCA 100
TCAACAGCCCTCATTTACAGTTGATTATAACTTGACGCTATGTGAGCTGAATGGCACAGATTGGTAGCTGGTGAATTTGACTGGGCAGTGGAGTGTTT 200
TGTCATTTTTCTGTGTTGACTCACATTGTCTCTATGGCGCCCTCACCACCAGCCATTTCTTGACGCAGTCGGTCTGGTCACTGTGTCTACCGCCGGC 300
TTTTCCCACGGCGGTATGTTCTGAGTAGCATCTACGCGGTCTGTGCCCTGGCTGCGTTGATTTGCTTCGTCAATTAGGTTTACGAAGAATTGCATGTCT 400
GGCGCTACTCATGTACCAGATATACCAACTTTCTCTGGACACTAAGGGCAGACTCTATCGTTGGCGGTCTGTCATCATAGAGAAAAGGGGTAAAGT 500
TGAGGTCTGAAGTCATCTGATCGACCTCAAGAGAGTTGTGCTTGATGGTTCGCGGCAACCCCTATAACCAAAATTTACGCGGAACAATGGGGTCGTCTC 600
TAG 603

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Figure 2 Nucleotide sequence of ORF5 gene

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MLGKCLTAGYCSSLFLFLWCIVPSWFVALASANSINSPHLQLIYNLTLCELNGTDWLAGFDFWAVECFVIFPVLTHIVSYGALTTSFLDAVGLVTVSTAG 100
FSHGRYVLSSIIYAVCALAALICFVIREFTKNCMSWRYSCTRYTNFLDTCGRLYRWRSLVIEKRGKVEVEGHLIDLKRVVLDGSAATPITKISAEQWGR 200
. 201

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Figure 3 Amino acid sequence of ORF5 gene

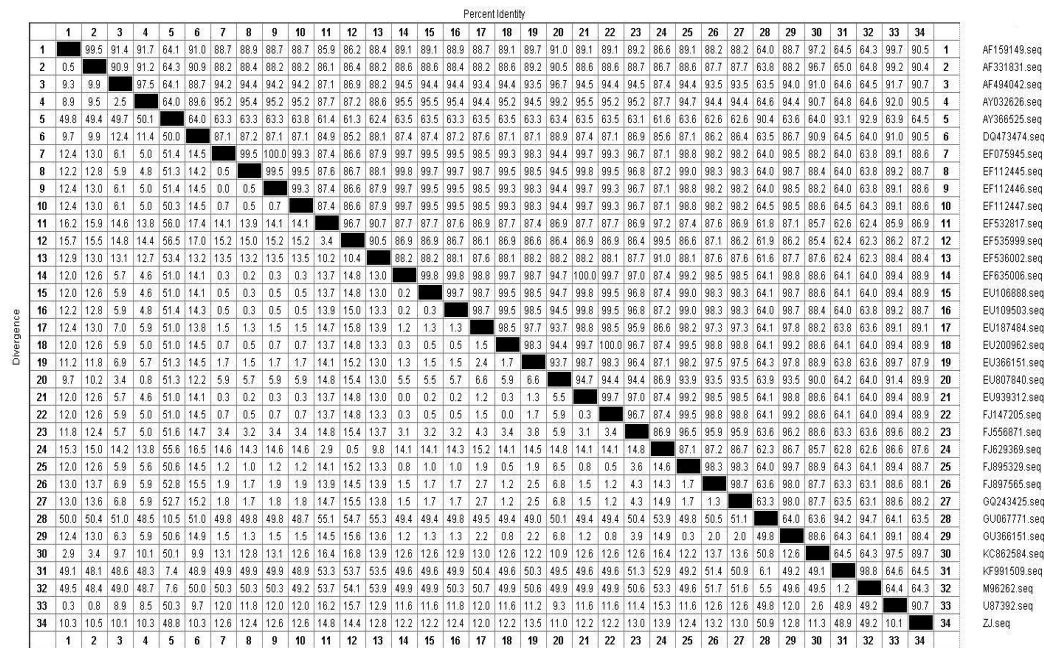
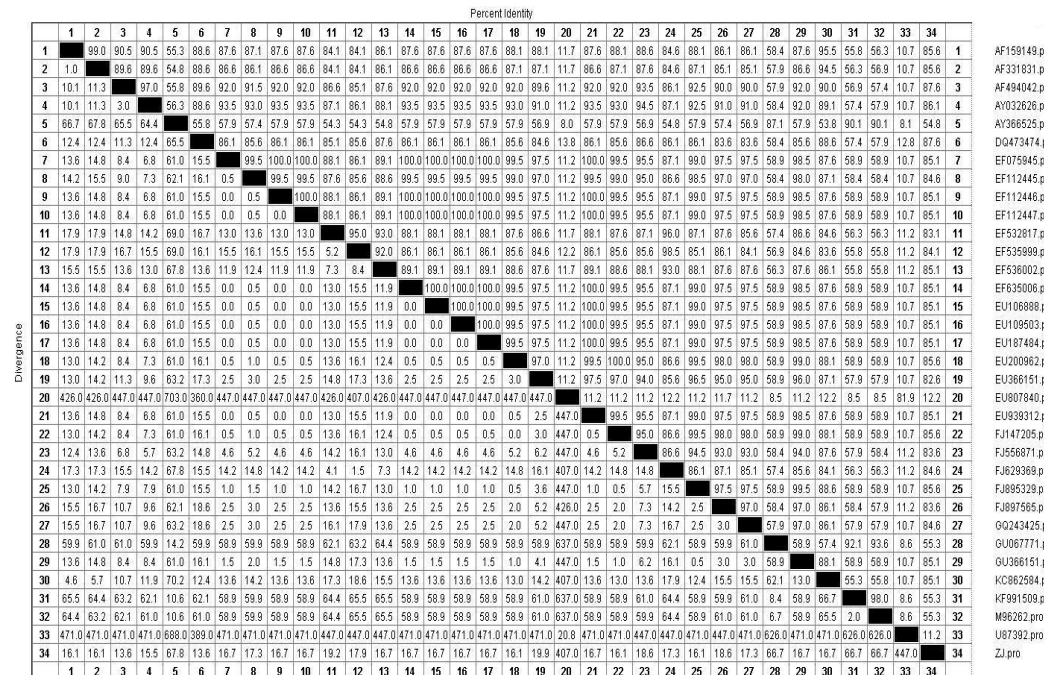


Figure 4 Homology comparison of ORF5 gene nucleotide sequence



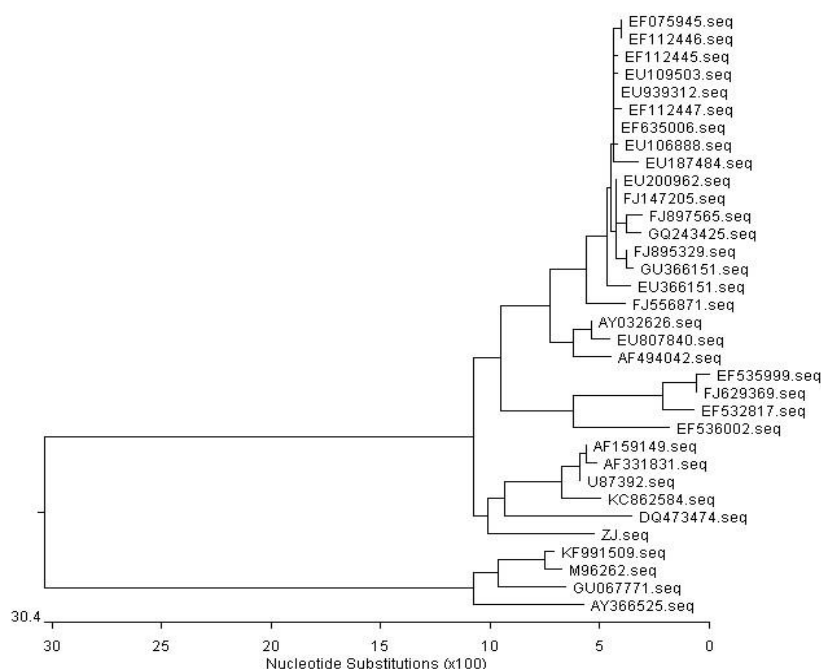


Figure 6 Phylogenetic tree of ORF5 gene

The nucleotide sequence homology of ZJ strain was 64.3% - 90.7% with other strain published in GenBank and 90.7% with the AF494042 strain of the American type. Therefore, we suggest that the isolate of ZJ strain belongs to American type. This study provides valuable reference for development of a vaccine based on GP5 protein.

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บทคัดย่อ

โคลนนิ่งและการวิเคราะห์ลำดับเบสของยีน ORF5 ของเชื้อไวรัสพาร์อาร์เอส สายพันธุ์ ZJ

Baocheng Hao^{1,3} Dengyuan Niu^{1,2} Xiaoyong Xing¹ Haitao Xiang¹ Fengqin Wen¹ Xiaoping Fu¹

Jianping Liang^{1,3} Yonghao Hu^{1*}

ไพรเมอร์พิเศษจำนวน 1 คู่ได้ถูกออกแบบและสังเคราะห์ตามลำดับเบสของเชื้อไวรัสพาร์อาร์เอส (PRRS) ซึ่งลำดับนี้ได้ถูกตีพิมพ์อยู่ใน GenBank ในปี ค.ศ. 2009 ฟาร์มสุกรแห่งหนึ่งในมณฑล Zhejiang สงสัยว่ามีสุกรที่ป่วยและเสียชีวิตที่แสดงอาการของ PRRS จึงได้ส่งตัวอย่างจำนวน 12 ตัวอย่าง (ม้าม ไต หรือต่อมน้ำเหลือง) เพื่อตรวจวินิจฉัยทางห้องปฏิบัติการ ตัวอย่างให้ผลบวกต่อเชื้อ PRRS ด้วยวิธี PCR ยีน ORF5 ได้ถูกเพิ่มจำนวนด้วยวิธี RT-PCR และโคลนเข้า pMD-18T vector ลำดับของสายพันธุ์ที่ได้อ้างอิงได้รับการวิเคราะห์โดยการ sequencing และเปรียบเทียบกับยีน ORF5 อื่นๆ ใน GenBank หลังจากนั้น โครงสร้างของยีน ORF5 ได้ถูกวิเคราะห์ ผลการทดลองได้แสดงให้เห็นว่าความยาวของยีนที่ถูกเพิ่มจำนวนนั้นมีขนาด 603 bp ซึ่งสามารถถอดรหัสได้กรดอะมิโนจำนวน 201 ชนิด เมื่อเปรียบเทียบตำแหน่งดังกล่าวกับสายพันธุ์อื่นของเชื้อไวรัส PRRS ความเหมือนกันของลำดับเบสอยู่ที่ 63.5-90.7% ของสายพันธุ์อื่นที่ตีพิมพ์ใน GenBank ความเหมือนกันของลำดับกรดอะมิโนมีค่า 11.2 - 87.6% และความเหมือนกันของลำดับสายพันธุ์เมื่อเทียบกับสายพันธุ์อเมริกา AF494042 มีค่า 90.7% จากผลการทดลองสรุปได้ว่าเชื้อสายพันธุ์ ZJ ที่แยกได้เป็นสายพันธุ์อเมริกา ยีน ORF5 ถอดรหัสได้เป็นโปรตีน GP5 ซึ่งเป็นโปรตีนหลักๆ ที่เกี่ยวกับโครงสร้างและภูมิคุ้มกันในการสร้าง neutralizing antibody ดังนั้น GP5 มีคุณสมบัติที่ดีในการเป็นโปรตีนเป้าหมายใหม่สำหรับการผลิตวัคซีนและชุดทดสอบในการวินิจฉัย การศึกษานี้ได้ให้ข้อมูลอ้างอิงที่มีค่าสำหรับการพัฒนาวัคซีนที่อาศัยโปรตีน GP5

คำสำคัญ: โคลนนิ่ง ORF5 ไวรัสพาร์อาร์เอส การวิเคราะห์ลำดับเบส

¹College of Veterinary Medicine, Gansu Agricultural University, Lanzhou 730070, PR China

²Institute of Animal sciences, Zhejiang academy of agricultural sciences, hangzhou 310021, PR China

³Key Laboratory of New Animal Drug Project, Gansu province/Key Laboratory of Veterinary Pharmaceuticals Discovery, Ministry of Agricultural /Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS , Lanzhou 730050, PR China

*ผู้รับผิดชอบบทความ E-mail: yhh0817@126.com