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Xiaoyong Xing
Haitao Xiang
Fengqin Wen

See next page for additional authors

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Authors
Baocheng Hao, Dengyuan Niu, Xiaoyong Xing, Haitao Xiang, Fengqin Wen, Xiaoping Fu, Jianping Liang, and Yonghao Hu

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

Baocheng Hao1,3 Dengyuan Niu1,2 Xiaoyong Xing1 Haitao Xiang1 Fengqin Wen1

Xiaoping Fu1 Jianping Liang1,3 Yonghao Hu*

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 Pharmaceutics Discovery, Ministry of Agricultural /Lanzhou Institute of Husbmdry 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 (Karnyuchuk 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) (Meulenberget 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 mitigated 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 (Ostrowskiet 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’-ggcttcataggtgctggggg aaatgcttgacc-3’ and the reverse primer was 5’-taagataaacagtgatgcatgcagcacgcttgc-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
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%, 88.2%, 87.6%, 88.7%, 88.1%, 86.3%, 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%, 83.1%, 84.1%, 85.1%, 85.1%, 85.1%, 85.1%, 85.1%, 86.2%, 12.2%, 85.1%, 85.6%, 84.6%, 85.6%, 83.6%, 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 a suitable target protein for gene engineering vaccine and diagnosis kit.
A lot of domestic and foreign research results showed that the ORF5 gene of PRRSV had strong immunogenicity. It could be used to study the vaccine of PRRSV. For example, Jiang et al. (2004) used attenuated Salmonella carrying a eukaryotic expression plasmid containing GP5 gene of PRRSV (pcDNA3-GP5) to induce specific GP5 immune in vivo. They detected GP5 protein in intestinal epithelium and neutralizing antibody in blood. This showed that oral vaccination could induce humoral immune response of PRRSV and Salmonella could be used for DNA vaccine by oral delivery.

The full ORF5 gene expression in Escherichia coli is low. It is becoming a bottleneck for further study of GP5 protein function. Studies showed that removal of the transmembrane region or strong hydrophobic sequence could significantly increase the amount of protein expression and obviously improve protein solubility (Hulst et al., 1993; Ji et al., 1993). Guo et al. (2004 and 2007) reported that the ORF5 gene which removing a transmembrane region of N end was expressed in Escherichia coli but the protein expression was not ideal.
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.

Acknowledgements

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References


บทคัดย่อ

こうนั้นและการวิเคราะห์ลำดับเบสของยีน ORF5 ของเชื้อไวรัสฟาร์อาร์เอสสายพันธุ์ ZJ

Baocheng Hao1,3 Dengyuan Niu1,2 Xiaoyong Xing1 Haitao Xiang1 Fengqin Wen1 Xiaoping Fu1

Jianping Liang1,3 Yonghao Hu1

โพรเมอร์พิเศษจำนวน 1 คู่ได้ถูกออกแบบและสังเคราะห์ตามลำดับเบสของเชื้อไวรัสฟาร์อาร์เอส (PRRS) ซึ่งล่าสุดนี้ได้ถูกพิมพ์ใน GenBank ในปี ค.ศ. 2009 ที่ร้านสุกุมแห่งหนึ่งในเขต Zhejiang ส่งบุคคลที่มีสุขภาพปกติเพื่อทดลองการตรวจ PRRS จึงได้ส่งตัวอย่างจำนวน 12 ตัวอย่าง (ม้า, โค, หรือตุ๊กตาเหลือง) เพื่อตรวจวินิจฉัยการติดเชื้อ PRRS ด้วยวิธี RT-PCR ยีน ORF5 ได้ถูกเพิ่มจำนวนตัวอย่าง RT-PCR และสังเคราะห์ปอดเข้ากับยีน ORF5 อื่น ๆ ใน GenBank หลังจากนั้น โครงสร้างของยีน ORF5 ได้ถูกตรวจสอบ ผลการทดลองได้แสดงให้เห็นว่าความยืดหยุ่นของยีนที่ถูกเพิ่มจำนวนนี้มีขนาด 603 bp ซึ่งสามารถตรวจพบได้ในธรรมชาติในจำนวน 201 ชนิด ซึ่งมีปริมาณเพียงตัวอย่าง ซึ่งนำไปสู่การเพิ่มลำดับของยีน PRRS ความเหมือนกันของลำดับเบสอยู่ที่ 63.5 - 90.7% ของสายพันธุ์อื่นที่ได้มาใน GenBank ความเหมือนกันของลำดับเกิดขึ้นในตัวอย่าง 11.2 - 87.6% และความเหมือนกันของลำดับสำยพันธุกรรมมีค่า 90.7% จากผลการทดสอบสุกุมได้เพื่อสุกุมเข้ากับยีน ORF5 ผลการทดสอบได้เป็นไปด้วยดี ซึ่งเป็นผลระดับสูง หลังจากนั้นได้เก็บข้อมูลเชิงพื้นฐานและการสังเคราะห์ neutralizing antibody ซึ่งมีคุณสมบัติที่ดีในการเป็นไปด้วยดีมีความหมายใหม่สู่การผลิตวัคซีนและชุดทดสอบในการวินิจฉัยการศึกษาเดียวกันจะส่งผลให้ผลการพัฒนาวัคซีนที่คาดว่าจะผลิต GPS