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Full Length Research Paper

Isolation and open reading frame 5 gene analysis of porcine reproductive and respiratory syndrome virus in Yunnan Province, China

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Two porcine reproductive and respiratory syndrome virus (PRRSV), respectively named YN-1 and YN-2 strains, were isolated by inoculation into Marc-145 cell. The two isolated strains induce Marc-145 cell stack together, pull net, form plaque and other typical lesions after 4 blind passages. With extracted viral RNA of fourth generation, reverse transcriptase (RT)-PCR based on open reading frame 5 (ORF5) gene showed that there was porcine reproductive and respiratory syndrome virus in Marc-145 cell of fourth generation. TCID50 of isolate measured by Reed-Muench method was $10^{-3.6}$/0.1 ml. Genetic evolution of ORF5 indicated that the two isolated strains were in a small branch with high identity of 99.5%. They were in a branch with Shandong strain JN-HS, Hennan-1 and Vietnam 347-T-KSA strain with identity of 99.2 to 99.8%. The two isolated strains were in a different branch with Ch-1a and VR-2332 strains having identity of 94.4 to 94.5%.

Key words: Porcine reproductive and respiratory syndrome virus (PRRSV), isolation, ORF5 gene, genetic evolution.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) also known as blue ear pig disease is mainly characterized by such symptoms as fever, anorexia, abortion, stillbirth, mummified, low earners and other reproductive disorders in sows and respiratory symptoms in piglets. It is also characterized by high mortality rates.

Protein GP5 (25 kDa) is the structural protein of PRRSV, which has been found to have most dramatic genetic mutation; the homology of North America and Europe strain is only 51 to 55% (Key et al., 2001). Whether *in- vivo* or *vitro*, neutralizing activity of PRRSV depends on GP5 antibody level (Plagemann, 2004; Wissink et al., 2003).

How to identify virulence of wild strain in molecular basis is not clear, although recently, 2 deletions found in NSP2 gene of PRRSV in China and Vietnam, reveals that NSP2 gene play an important role in the virulence (Zhou et al., 2008; Li et al., 2007; Tian et al., 2007; Feng et al., 2008). However, reverse genetic operation shows that there is no direct connection between virulence of virus and gene deletion (Zhou et al., 2009). So, the exploration of virulent gene reaches an impasse again.

So far, blue ear pig disease occasionally occurred in Yunnan province, resulting in huge economic losses. Therefore, the study of the isolation and identification of biological characteristics of porcine reproductive and respiratory syndrome virus and the analysis of the molecular genetic characteristics would be of great significance for prevention and control of reproductive and respiratory syndrome in Yunnan province in China.

MATERIALS AND METHODS

Isolation of porcine reproductive and respiratory syndrome virus

Cells of the MARC-145 line- a subclone of the African green

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monkey kidney epithelial cell line that is highly permissive for PRRSV replication, were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco Corporation, NY) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories Inc., South Logan, UT) at 37°C under 5% CO_2.

Suspected lung tissue was homogenized with grinding stones, and centrifuge at 4,000 rpm for 10 min, then the supernatant was sterilized with 0.22 µm filters. One millliter (1 ml) filtrate was inoculated in Marc-145 cells at 37°C for 1 h adsorption. The inoculation fluid was removed, Marc-145 cell monolayer was washed with 199 cell culture medium for 3 times, then 15 ml 199 cell culture medium containing 2% fetal bovine serum was added, and cultured for six days until the virus was harvested. It was freezed and thawed three times at -20°C and 1 ml of the supernatant was inoculated into Marc-145 cell monolayer; fourth generation of blind passage was got through the same method and cytopathic effect was (CPE) observed every day.

Table 1. The PRRSV isolates used in this study.

Strain	Accession no.
Ch-1a	AY032626
VR-2332	AY150564
JN-HS	HM016158
347-T-KS	AB588636
Henan-1	EU200962
BJsy06	EU097707
GS2008	EU880431
JXM80	GQ499196
HNly	EF471928
Jiangxi-3	EU200961
JXA1	EF112445
BJSY-1	FJ950744
WUH1	EU187484
JXM20	GQ499193
SD-CXA	GQ359108
171-NA	AB588638
JSyx	EU939312
JX143	EU708726
HNsf	EF471930
Jiangxi-2	EF398046

Identification of cultures of virus nucleic acid

Viral RNA of fourth generation was extracted by column viral RNA extraction kit (TransGen Biotech, China), and PRRSV nucleic acid was detected with the first pair of primers (PF and PR) by One Step RT-PCR Kit (TaKaRa, Japan). Reaction conditions for one step RT-PCR reaction is 50°C 30 min; 94°C 2 min; 94°C 30 s; 60°C 40 s; 72°C 45 s; 30 cycles, 72°C for 5 min. PF: 5'-AGCCTGTCTTTTGCCATTCT-3', PR: 5'-CTTTTGTGGAGCCGTGCTATC-3'. $TCID_{50}$ (Median tissue culture infective dose) of virus isolated**.**

199 cell culture medium (without newborn calf serum) was diluted to 10^{-1} ~ 10^{-6} (a total of 6 dilutions), each dilution was inoculated to Marc-145 cell monolayers in 96 well cell culture plates and inoculated with 8 holes, with adsorption at 37°C for 1 h, the inoculation fluid was removed, Marc-145 cell monolayer was washed with 199 cell culture medium for 3 times, then 200 µL 199 cell culture medium (containing 2% fetal bovine serum) was added at 37°C under 5% CO_2 for 6 days until the virus was received. Cytopathic effect were observed every day, the amount of virus in infected tissue half TCID50 = $10^{-3.6}$ was calculated according to Reed-Muench method.

Amplification of ORF5 gene

PRRSV YN-1 and YN-2 strain virus RNA were extracted by column viral RNA extraction kit, ORF5 gene was amplified by one step RT-PCR Kit (TaKaRa, Japan), One Step RT-PCR reaction conditions is as shown above.

Sequencing and analysis of the ORF5 gene

The PCR products of PRRSV YN-1 and YN-2 strain virus ORF5 gene were purified using PCR purification kit (Axygen, USA) and cloned into the pMD19-Tvector (TaKaRa, Japan). Finally, the plasmid was sequenced by TaKaRa. The ORF5 gene of the two strains YN-1 and YN-2 and 21 reference sequences download from GenBank (Table 1) was compared with the Lasergene sequence analysis software package (DNASTAR Inc., Madison,WI).

RESULTS

Virus isolation

Cell shedding, rounding and vacuolization appeared on Marc-145 cells at the fourth day of 3rd blind passages. Apparent cytopathic effect, rounding and gathering, block off and formation of holes was observed on Marc-145 cells at the fourth day of 4th blind passages. In contrast, there was no lesion on cells of negative control.

Identification of viral nucleic acid

Amplification of the ORF5 gene with the first pair of primers

It was known that the molecular weight of highly pathogenic PRRSV nucleic acid was about 600 bp. The 4th generation of virus cultures and positive control amplified fragments of the same size (Figure 1) shows that the porcine reproductive and respiratory syndrome virus has been isolated and cultured by the 4th generation of the virus culture. In contrast, there was no fragment amplified by negative control which proved that the PCR method steps were correct.

Determination of the virus TCID50

The virus TCID50 was calculated by Reed-Muench's method and found to be $10^{-3.6}$/0.1 ml. When inoculated with 0.1 ml/hole, 50% of the cell produced CPE.

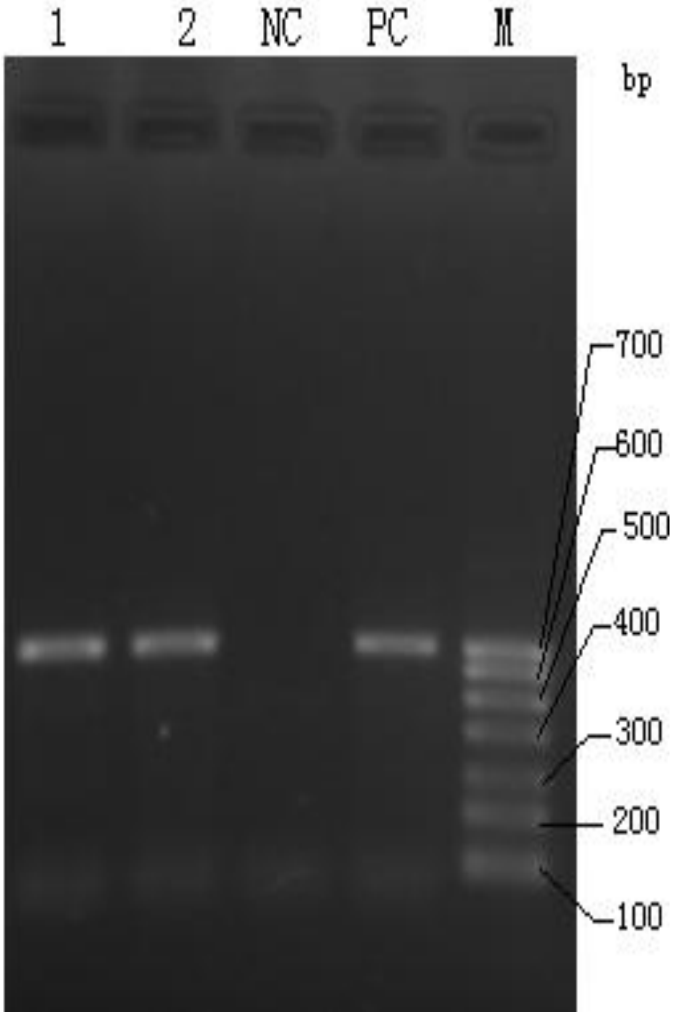


Figure 1. Identification of the ORF5 gene. Lane 1: YN-1, lane 2:YN-2, NC: negative control, PC: positive control, lane M: DNA marker.

Genetic characteristics of ORF5 gene

Phylogenetic tree of the ORF5 gene sequences was generated by DNASTAR (Figure 2). Two strains of PRRSV isolates were in the same small branch of the phylogenetic tree, and nucleotide homology between the two strains was nearly 99.5% (Figure 3).

The two isolated classical strains Ch-1a and VR-2332 were of two different branches, and nucleotide homology was only 94.4%. The two strains and other virulent domestic strains (such as JXA1) were in the same branch, and nucleotide homology was 98 to 99%.

The two isolates, the Shandong strain JN-HS, Henan strain Henan-1, and the Vietnam strain 347-T-KSA were in the same small branch, the nucleotide homology was 99.2 to 99.8%, especially the Shandong strain JN-HS, and nucleotide homology was up to 99.8%.

There was also closer genetic distance among the two strains: Vietnam strain 347-T-KS and 171-NA, and nucleotide homology was 98.3 to 99.5%, particularly, the Vietnam strain 347-T-KS nucleotide homology was 99.5%.

DISCUSSION

Monkey embryonic kidney cells MA-104, Marc145 (an MA-104 clone from fetus monkey kidney) (Kim et al., 1993) and pig alveolar macrophages (PAMs) are the three main cell lines for the replication, isolation and identification of PRRSV. Studies show that strain VR-2332 grew readily in MA104 cells [maximum titer, 10^7 TCID50 per milliliter at 30 h] but not in PAMs (10^2 TCID50/mL at 72 h) (de Abin et al., 2009).

In this study, the two PRRSV viruses YN-1 and YN-2 was successfully separated by Marc-145 cells (an MA-104 clone from fetus monkey kidney) (Kim et al., 1993). Cytopathic effect appeared at the fourth day of 3rd blind passages. Apparently cytopathic effect was observed on Marc-145 cells on the fourth day of 4th blind passages, rounding, gathering, block off and formation of holes. So the two isolates, consistent with previous domestic reports, belong to American strain.

The recent research on PRRSV focus on genetic variation, particularly the variation of ORF5, it was reported that evolution of PRRSV was faster than other RNA virus, and high rate of PRRSV evolution is closely related to the outbreak of porcine reproductive and respiratory syndrome in China (Song et al., 2010).

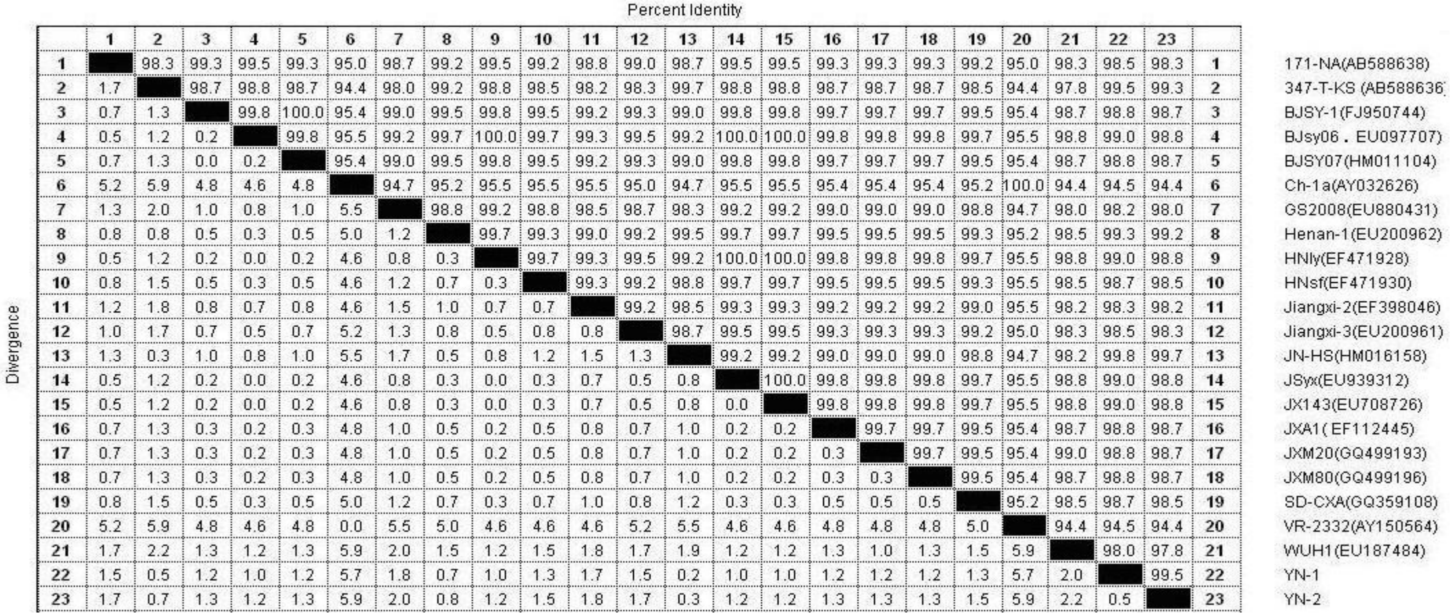
Wesley et al. (1998) reported that wild strain and vaccine strain can be identified according to the amino acid sequence analysis of ORF5, because of the 137th amino acid residue of wild strain ORF5 mutated, Ala of vaccine strain was replaced by Ser, 137th amino acids of the 2 isolates are Ser in this study, which is consistent with the molecular characteristics of wild strains.

Nucleotide homology and genetic characteristics analysis of ORF5 showed that 2 strains isolated from PRRSV virus YN-1 and YN-2 was in the same small branch of the phylogenetic tree (Figure 3), and nucleotide homology was up to 99.5%.

The two classical strains, internal strains Ch-1a and VR-2332 are in two different branches, their genetic distances are furthest, nucleotide homology is only 94.4%, and the nucleotide homology of the two strains and the domestic strains of other nine provinces are 98 to 99%, which is consistent with the domestic report.

However, the difference is that nucleotide homology is 99.5% between YN-1 isolates and Vietnam strains 347-T-KS and homology 98.5% with the Vietnam strain 171-NA; nucleotide homology is 99.3% between strain YN-2 and Vietnam strains 347-T-KS and homology is 98.3% with the Vietnam strain 171-NA.

The discovery provides a reference value for prevention and control of the porcine reproductive and respiratory syndrome disease in border areas of Yunnan.



Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
1	■	98.3	99.3	99.5	99.3	95.0	98.7	99.2	99.5	99.2	98.8	99.0	98.7	99.5	99.5	99.3	99.3	99.3	99.2	95.0	98.3	98.5	98.3	1	171-NA(AB588638)
2	1.7	■	98.7	98.8	98.7	94.4	98.0	99.2	98.8	98.5	98.2	98.3	99.7	98.8	98.8	98.7	98.7	98.7	98.5	94.4	97.8	99.5	99.3	2	347-T-KS (AB588636
3	0.7	1.3	■	99.8	100.0	95.4	99.0	99.5	99.8	99.5	99.2	99.3	99.0	99.8	99.8	99.7	99.7	99.7	99.5	95.4	98.7	98.8	98.7	3	BJSY-1(FJ950744)
4	0.5	1.2	0.2	■	99.8	95.5	99.2	99.7	100.0	99.7	99.3	99.5	99.2	100.0	100.0	99.8	99.8	99.8	99.7	95.5	98.8	99.0	98.8	4	BJsy06 . EU097707)
5	0.7	1.3	0.0	0.2	■	95.4	99.0	99.5	99.8	99.5	99.2	99.3	99.0	99.8	99.8	99.7	99.7	99.7	99.5	95.4	98.7	98.8	98.7	5	BJSY07(HM011104)
6	5.2	5.9	4.8	4.6	4.8	■	94.7	95.2	95.5	95.5	95.5	95.0	94.7	95.5	95.5	95.4	95.4	95.4	95.2	100.0	94.4	94.5	94.4	6	Ch-1a(AY032626)
7	1.3	2.0	1.0	0.8	1.0	5.5	■	98.8	99.2	98.8	98.5	98.7	98.3	99.2	99.2	99.0	99.0	99.0	98.8	94.7	98.0	98.2	98.0	7	GS2008(EU880431)
8	0.8	0.8	0.5	0.3	0.5	5.0	1.2	■	99.7	99.3	99.0	99.2	99.5	99.7	99.7	99.5	99.5	99.5	99.3	95.2	98.5	99.3	99.2	8	Henan-1(EU200962)
9	0.5	1.2	0.2	0.0	0.2	4.6	0.8	0.3	■	99.7	99.3	99.5	99.2	100.0	100.0	99.8	99.8	99.8	99.7	95.5	98.8	99.0	98.8	9	HNly(EF471928)
10	0.8	1.5	0.5	0.3	0.5	4.6	1.2	0.7	0.3	■	99.3	99.2	98.8	99.7	99.7	99.5	99.5	99.5	99.3	95.5	98.5	98.7	98.5	10	HNsf(EF471930)
11	1.2	1.8	0.8	0.7	0.8	4.6	1.5	1.0	0.7	0.7	■	99.2	98.5	99.3	99.3	99.2	99.2	99.2	99.0	95.5	98.2	98.3	98.2	11	Jiangxi-2(EF398046)
12	1.0	1.7	0.7	0.5	0.7	5.2	1.3	0.8	0.5	0.8	0.8	■	98.7	99.5	99.5	99.3	99.3	99.3	99.2	95.0	98.3	98.5	98.3	12	Jiangxi-3(EU200961)
13	1.3	0.3	1.0	0.8	1.0	5.5	1.7	0.5	0.8	1.2	1.5	1.3	■	99.2	99.2	99.0	99.0	99.0	98.8	94.7	98.2	99.8	99.7	13	JN-HS(HM016158)
14	0.5	1.2	0.2	0.0	0.2	4.6	0.8	0.3	0.0	0.3	0.7	0.5	0.8	■	100.0	99.8	99.8	99.8	99.7	95.5	98.8	99.0	98.8	14	JSyx(EU939312)
15	0.5	1.2	0.2	0.0	0.2	4.6	0.8	0.3	0.0	0.3	0.7	0.5	0.8	0.0	■	99.8	99.8	99.8	99.7	95.5	98.8	99.0	98.8	15	JX143(EU708726)
16	0.7	1.3	0.3	0.2	0.3	4.8	1.0	0.5	0.2	0.5	0.8	0.7	1.0	0.2	0.2	■	99.7	99.7	99.5	95.4	98.7	98.8	98.7	16	JXA1(EF112445)
17	0.7	1.3	0.3	0.2	0.3	4.8	1.0	0.5	0.2	0.5	0.8	0.7	1.0	0.2	0.2	0.3	■	99.7	99.5	95.4	99.0	98.8	98.7	17	JXM20(GQ499193)
18	0.7	1.3	0.3	0.2	0.3	4.8	1.0	0.5	0.2	0.5	0.8	0.7	1.0	0.2	0.2	0.3	0.3	■	99.5	95.4	98.7	98.8	98.7	18	JXM80(GQ499196)
19	0.8	1.5	0.5	0.3	0.5	5.0	1.2	0.7	0.3	0.7	1.0	0.8	1.2	0.3	0.3	0.5	0.5	0.5	■	95.2	98.5	98.7	98.5	19	SD-CXA(GQ359108)
20	5.2	5.9	4.8	4.6	4.8	0.0	5.5	5.0	4.6	4.6	4.6	5.2	5.5	4.6	4.6	4.8	4.8	4.8	5.0	■	94.4	94.5	94.4	20	VR-2332(AY150564)
21	1.7	2.2	1.3	1.2	1.3	5.9	2.0	1.5	1.2	1.5	1.8	1.7	1.9	1.2	1.2	1.3	1.0	1.3	1.5	5.9	■	98.0	97.8	21	WUH1(EU187484)
22	1.5	0.5	1.2	1.0	1.2	5.7	1.8	0.7	1.0	1.3	1.7	1.5	0.2	1.0	1.0	1.2	1.2	1.2	1.3	5.7	2.0	■	99.5	22	YN-1
23	1.7	0.7	1.3	1.2	1.3	5.9	2.0	0.8	1.2	1.5	1.8	1.7	0.3	1.2	1.2	1.3	1.3	1.3	1.5	5.9	2.2	0.5	■	23	YN-2

Figure 2. The nucleotide homology of PRRSV ORF5 analysis.

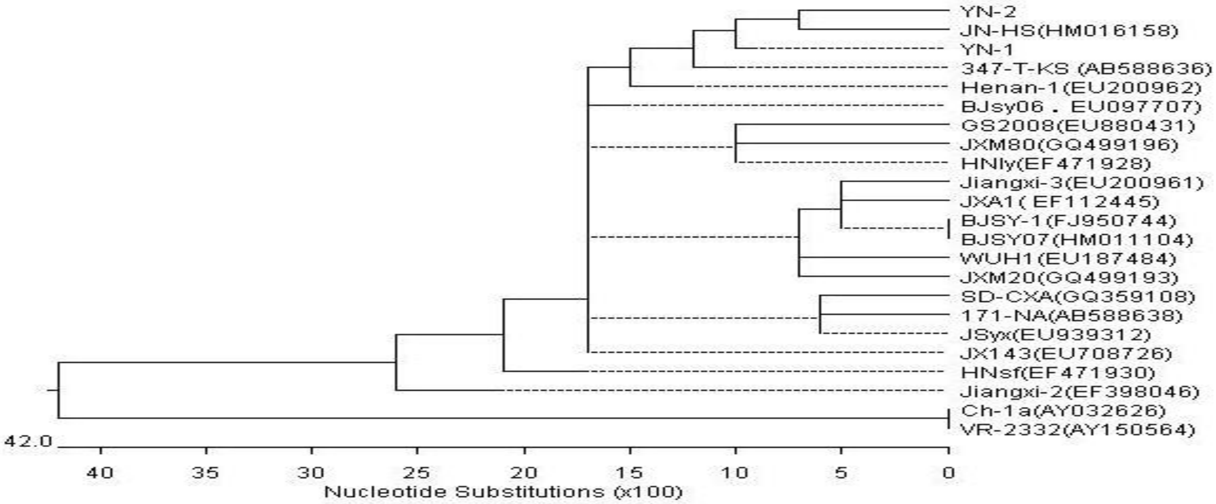


Figure 3. Unrooted phylogenetic tree of PRRSV ORF5.

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