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Evolution of quasispecies diversity for porcine reproductive and respiratory syndrome virus under antibody selective pressure

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To study the quasispecies diversity of porcine reproductive and respiratory syndrome virus (PRRSV), open reading frame 5 (*ORF5*) of strain SD0612 was amplified and cloned. Sixty clones of *ORF5* were sequenced and analyzed with DNAStar software. Nucleic acid sequence homology was 97.7%–100%, with 78 mutations observed. Among these 60 clones, the sequences of 17 clones were identical and recognized as the dominant quasispecies of strain SD0612. Evolution of SD0612 quasispecies diversity under antibody selective pressure was also studied. SD0612 was passed continuously in the Marc-145 cell line over 40 passages in 6 independent lineages. SD0612 antiserum was not added to lineage A, B, and C cultures; however, antiserum was added to culture medium for lineages D, E, and F. PRRSV *ORF5* was then amplified, cloned, and sequenced from each of the 6 lineages, designated as A40–F40. F40 was further passed in Marc-145 cells using 6 independent lineages with or without F40 antiserum for another 40 passages. *ORF5* from the 6 newly-derived virus lineages, which we designated as a40–f40, were amplified, cloned and sequenced. The proportion of dominant quasispecies increased with passage number in cell cultures supplemented with antibodies, but decreased when antibodies were lacking. Our work has demonstrated a diversity of quasispecies for *ORF5* in PRRSV SD0612. Antibody selective pressure was able to significantly influence quasispecies diversity and promote a dominant quasispecies that was able to evade immune reactions.

porcine reproductive and respiratory syndrome virus (PRRSV), antibody selective pressure, quasispecies diversity, *ORF5*, *ORF6*

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Porcine reproductive and respiratory syndrome (PRRS) is a common disease that affects the reproductive and respiratory systems of pigs. This results in large worldwide economic losses to the pig industry. PRRS virus (PRRSV) is a single-stranded positive sense RNA virus. Its genome is around 15 kb, encoding 8 open reading frames (ORFs) [1–3]. Of the many PRRSV structural proteins, GP5 which is encoded by *ORF5*, is considered the most important antigen. Mutations in *ORF5* occur rapidly in infected pigs, and it is thought to be the most important reference gene with respect to PRRSV evolution [1–3].

The first report of atypical PRRS in China was in 2006. It was characterized by high fever, morbidity and mortality, which spread rapidly in pig breeding areas and caused a pandemic outbreak. Studies have shown that the main pathogen was a highly pathogenic PRRSV variant (HP-PRRSV) characterized by a 90 bp deletion in the *NSP2* gene [4–6]. Whole genome comparisons indicated that *ORF5* of HP-PRRSV isolates shared a high degree of homology; however, there were some mutations evident, even in strains isolated from the same area [4–6]. It was speculated that this may have been due to the response of different quasispecies of PRRSV isolates.

In its interaction with the host, the virus continues to

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evolve and mutate. Over the last 10 years, increased attention has been given to the influences of antibody selective pressures on the evolution of viruses. Our laboratory successfully studied the effects of immune selective pressures on the evolution of certain avian RNA viruses, such as avian leukosis virus (ALV) [7], the H9N2 subtype of avian influenza virus (H9-AIV) [8], and Newcastle disease virus (NDV) [9]. In all these studies, effects of antibody-mediated immune selective pressures on the evolution of certain specific antigen genes related to protective immunity were documented. The influence of antibody-mediated selective pressures on *ORF3*, 4, and 5 mutations in PRRSV was also studied [10]. In that study we attempted to identify amino acid sites that had been mutated. In this study, we characterized the evolutionary process of quasispecies diversity for HP-PRRSV SD0612 *ORF5*.

1 Materials and methods

1.1 Cells and viruses

Marc-145 cell lines were used to culture PRRSV. Dulbecco's modified Eagle's medium (DMEM, pH 7.2; Invitrogen; Carlsbad, California, USA) was used as basic medium. Growth medium was DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen). Maintenance medium was DMEM containing 2% FBS. Penicillin and streptomycin (Sigma-Aldrich, Saint Louis, Missouri, USA) were added to the media at a final concentration of 100 μg mL^{-1}. Cell cultures were incubated at 37°C/5% CO_2.

In 2006, PRRSV strain SD0612 was isolated from a breeder farm containing pigs displaying typical PRRS symptoms and lesions [10]. Sequence analysis indicated a 30 amino acid deletion in the NSP2 protein. The same deletion was previously reported in other highly pathogenic strains [4]. SD0612 was highly pathogenic and caused 100% mortality when inoculated into 2-month-old antibody-negative pigs. SD0612 was amplified, titered, and stored at −80°C until required.

1.2 Preparation of PRRSV antiserum

To prepare the specific high-titer antiserum against PRRSV, two 4-month-old pigs lacking specific antibodies were subjected to intramuscular injections (2×10^4 50% tissue culture infectious dose ($TCID_{50}$)) of SD0612. Serum samples were collected 35 days and 42 days post-inoculation. Serum was filtered through 0.22-μm pore membranes (Millipore, Billerica, Massachusetts, USA) and serum neutralization titers determined. The serum with the highest SN titer was used in this study. Filtered serum was aliquoted into sterilized 1.5 mL microcentrifuge tubes and stored at −40°C until required. One vial of serum was used to inoculate Marc-145 cells and virus replication assessed.

1.3 Determination of a PRRSV antiserum concentration that inhibits viral replication

To study the influence of antibody selective pressures on quasispecies diversity of PRRSV, it was important to use suitable concentrations of PRRSV antiserum that would partially inhibit viral replication. Marc-145 cells were seeded into 6-well plates. When monolayers had formed, cells were inoculated with 200 $TCID_{50}$ of SD0612. Two hours after inoculation the medium in each well was replaced with maintenance medium and PRRSV antiserum added into the 6 wells (diluted 1:5, 1:10, 1:25, 1:50, 1:100, and 1:200 relative to the volume of maintenance medium in the well). Cytopathic effects (CPE) of PRRSV infection were observed using a microscope at 12, 24, 36, 48, 72, and 120 h post-inoculation. Each experiment was repeated three times.

1.4 Continuous viral passage in cell cultures of different lineages

In the first round of continuous passage, Marc-145 cells were seeded into 6-well plates, with wells allocated to one of two groups (groups I and II). The medium in wells A, B, and C of group I wells was supplemented with 10% pig serum negative for PRRSV over 40 passages. The medium in wells D, E, and F of group II wells was supplemented with 10% PRRSV SD0612 antiserum over 40 passages. At the start of the experiment all wells were inoculated with 200 $TCID_{50}$ of SD0612 and cultures incubated at 37°C/5% CO_2. When 70%–80% of the Marc-145 monolayer showed CPE 3–4 days later. Supernatants from each well were collected, designated as A1, B1, C1, D1, E1 and F1, and stored at −80°C. Fresh cultures of Marc-145 cells in 6-well plates were prepared and each well inoculated with 20 μL of supernatant from A1, B1, C1, D1, E1, or F1 and incubated at 37°C/5% CO_2. When 70%–80% of cells exhibited CPE, supernatants were collected and designated as A2, B2, C2, D2, E2, or F2. This was repeated for 40 passages, until supernatants A40–F40 were collected.

To further characterize mutants generated from the above passages, a second round of continuous passage was conducted. Convalescent serum from the F40 mutant derived from SD0612 was prepared in pigs as in section 1.2. F40 was further passed through 6 lineages (a, b, c, d, e, and f) of Marc-145 cells with or without F40 antiserum. Supernatants from each round of passage were collected until passage 40 (a40–f40).

1.5 Amplification and sequencing of *ORF5* and *ORF6*

Cells infected with SD0612 were harvested when 70%–80% of the Marc-145 monolayer showed CPE. Viral genomic RNA was extracted from infected Marc-145 cells using a Viral RNA Kit (Omega Bio-Tek, Inc., Norcross, Georgia,

USA). *ORF5* was amplified by reverse transcription polymerase chain reaction (RT-PCR) with primers ORF5-F (5'-AGCCTGTCTTTTTGCCATCCCT-3') and ORF5-R (5'-CTTTTGTGGAGCCGTGCTATC-3'). The thermal cycling conditions we used have been reported previously [11]. To exclude sequencing errors, the highly conserved PRRSV ORF6 was amplified by RT-PCR with primers ORF6-F (5'-GCGGTCGCCCATCATTGTG-3') and ORF6-R (5'-GCTGGCCATTCCCCTTCTTTTT-3'). The *ORF5* amplicon was 682 bp, and the ORF6 amplicon was 689 bp.

Amplicons were purified after gel electrophoresis using an EZNA Gel Extraction Kit (Omega) and cloned into the pMD-18T plasmid vector (Takara Bio Inc., Tokyo, Japan). Transformed competent *Escherichia coli* DH5α colonies were screened and identified. All positive clones were sequenced by a commercial sequencing service and sequence data analyzed with DNAstar software. More than 25 clones from each amplicon were sequenced and analyzed.

2 Results

2.1 Incomplete inhibition of viral replication by SD0612 antiserum

At 48 h post-inoculation with SD0612, CPE was observed in wells where the final antiserum dilutions were 1:50, 1:100, and 1:200. Most cells had died by 72 h post-inoculation. CPE was not detected at the 1:5 dilution. The Marc-145 monolayer appeared normal at 120 h post-inoculation, indicating that virus replication was completely inhibited. Appearance of CPE in wells containing antisera at dilutions of 1:10 and 1:25 was delayed, indicating that virus replication was partially inhibited. Based on these results, PRRSV antiserum was added to cell cultures at a dilution of 1:10 during continuous passage.

2.2 Analysis of quasispecies diversity of SD0612

Sixty positive *ORF5* clones from the original SD0612 strain were sequenced. Nucleic acid homologies ranged from 97.7% to 100%, with 78 nucleotide mutation sites found. Among these 60 clones, 17 clones contained identical sequences and were recognized as the dominant SD0612 quasispecies. Other quasispecies sequences shared 94.5%–99.5% homology with the dominant quasispecies. For *ORF6*, 54 positive clones were sequenced and nucleic acid sequence homologies ranged from 99.2% to 100%. Among these 54 clones, 46 had identical sequences, with only 8 mutation sites observed. Taken together, these results indicate that the quasispecies diversity of PRRSV *ORF6* was much lower than that for *ORF5*. The sequence differences between different clones for *ORF5* indicated that HP-PRRSV SD0612 was composed of many different quasispecies, despite the fact that these quasispecies shared a high homology.

2.3 Evolution of *ORF5* quasispecies diversity for SD0612 and F40

Tables 1 and 2 summarize data regarding the evolution of quasispecies diversity of *ORF5* during two rounds of continuous passage in cell culture, with or without specific antibodies. In Table 1, data from 6 lineages over 2 rounds of passage are shown. The proportion of dominant quasispecies rose from 28.33% in the original SD0612 stock to 25.0%–39.47% in lineages D40, E40, and F40 after 40 passages with SD0692 antiserum. The homology among all clones increased from 97.7% to 100%, from 98.0% to 100%, from 99.0% to 100%, and from 99.0% to 100% for SD0612, D40, E40 and F40, respectively. The proportion of dominant quasispecies for *ORF5* decreased to 11.11%–17.85% after 40 passages without antiserum. The homology among different clones was also decreased compared with the original SD0612. During another 40 passages, the proportion of dominant quasispecies rose to 33.33%–44.44% in d40, e40, and f40 when F40 antiserum was present. Homology increased to 99.0%–100% for all 3 lineages; however, the proportion of dominant quasispecies in the 3 lineages lacking antiserum decreased to 0–18.51%. All 27 clones sequenced from one PCR product are different from each other (Table 1) with the lowest level of homology at 97.8% for b40. Table 2 summarizes the data for the 3 lineages with and without antiserum, clearly showing the influence of antibody-mediated selective pressure on PRRSV evolution.

3 Discussion

PRRSV is one of the most significant pathogens causing reproductive disorders in pigs. It has been constantly mutating and evolving since it was first identified. GP5, encoded by *ORF5*, is the major antigenic protein of PRRSV. It quickly mutates and is probably the most important target for studying the evolution or quasispecies diversity of PRRSV [1–3].

An atypical PRRS outbreak occurred in China in 2006 and spread to more than 10 provinces, resulting in approximately 400000 fatal cases. PRRSV field strains with high pathogenicity were isolated and it was noticed that the variants had a 30 amino acid deletion in NSP2 [4–6]. When the sequences of the genomes were compared, variation in those isolated from 2006 and the strain isolated in 1997 were mainly seen in GP5. Sequence analysis also found that *ORF5* in the JXA1, HuN4, and SY0608 variants demonstrated a high degree of homology, but were not identical, even for strains isolated in the same area. This indicated that perhaps antibodies or cell-mediated immunity against PRRSV in vaccinated herds promoted antigenic evolution.

Quasispecies of viruses have been correlated with gene duplication. It is thought that the mutation rate of each nucleotide in the hemagglutinin (*HA*) gene of AIV-H9N2 is

Table 1 Evolution of quasispecies diversity for PRRSV SD0612 during passage with and without antibodies[a)]

Group		Lineages	Total clones sequenced[a]	Clones with identical sequence[b]	Clone numbers with bases different from each other[c]	Sequence homology among all clones in the lineage[d]	Mutation points between all clones in the lineage[e]	Ratios of the dominant quasispecies clone[f]
First passage round	Without anti-SD016 sera	A40	27	3	24	98.5%–100%	35	11.11%
		B40	27	3	24	98.3%–100%	27	11.11%
		C40	28	5	23	98.7%–100%	46	17.85%
	With anti-SD016 sera	D40	28	7	21	98.8%–100%	22	25.00%
		E40	28	9	19	99.0%–100%	20	32.14%
		F40	38	15	23	99.0%–100%	29	39.47%
Second passage round	Without anti-F40 sera	a40	27	5	22	99.0%–100%	34	18.51%
		b40	27	0	27	97.8%–99.8%	49	0
		c40	28	3	25	98.5%–100%	39	10.71%
	With anti-F40 sera	d40	27	9	18	99.0%–100%	24	33.33%
		e40	36	16	20	99.0%–100%	36	44.44%
		f40	27	9	18	99.0%–100%	26	33.33%

a) Column a lists the number of total clones obtained; the clone number listed in column b indicates the clones whose sequences were identical; the clone number listed in column c indicates the clones whose sequences were different; sequence homology in column d and mutation points in column e are the comparisons between all clones in column a. The percentages of the dominant quasispecies comes from the proportion of the clone number listed in column b to total clones.

Table 2 A comparison of the proportions of dominant PRRSV *ORF5* quasispecies following two rounds of continuous passage with or without antibodies[a)]

Groups		Total clones sequenced[a]	Clones with identical sequence[b]	Ratios of the dominant quasispecies[c]
SD0612 original strain		60	17	28.33%
First passage round	Without anti-SD016 sera	82	11	13.41%
	With anti-SD016 sera	94	31	32.98%
Second passage round	Without anti-F40 sera	82	8	9.76%
	With anti-F40 sera	90	34	37.78%

The ratio of the dominant quasispecies comes from the proportion of the clone number listed in column b to total clones listed in column a.

2×10^{-3} for each copy cycle. This means that a base mutation is predicted to occur every duplication cycle [12]. RNA viruses *in vitro* and *in vivo* are actually a population comprising different quasispecies with some differences in their sequences. In this study, 60 *ORF5* clones amplified from an original stock of HP-PRRSV SD0612 were sequenced and compared. Sequence homology was 97.7%–100%, with only 17 clones having identical sequences. The remaining clones contained various mutations located over 78 different sites. *ORF6* was more conserved, with homologies of 99.2%–100% in 54 clones, and 85% (46/54) of clones having identical sequences. Additionally we identified only 8 different mutation sites among the 54 clones. Taken together, our results suggest that *ORF5* was more important than *ORF6* for determination of PRRSV quasispecies diversity.

Because of the lack of a correction mechanism for RNA polymerases, RNA viruses are prone to errors during replication. It is assumed that amino acid changes at certain sites of envelope glycoproteins help mutated viruses evade the neutralizing activity of specific antibodies. Mutants resistant to neutralizing antibodies should be able to replicate faster than parental viruses. These gradually become the dominant quasispecies in the virus population [13]. Various selective pressures in nature, especially those related to the immune system, not only accelerate mutations of viruses but also help some virus variants become the dominant population. An example of this is the human immunodeficiency virus (HIV) envelope glycoprotein genes. They have high frequencies of mutation, with the V3 loop having mutation rates as high as 50% [14]. The V3 loop is closely associated with cell tropism, replication kinetics and pathogenicity of HIV. Mutations in the loop help the virus escape attacks by cytotoxic T cells or neutralizing antibodies [15]. We have successfully studied the effects of immune selective pressures on the evolution of chicken viruses [6–8]. In all studies, the effects of antibody-mediated immune selective pressures on the evolution of certain antigen-related genes were clearly demonstrated. The role for antibody selective pressures in the generation of quasispecies diversity was clearly shown over two prolonged passages with and without antibodies in the present study. Our results demonstrate that passaging with a specific antiserum could promote population of a dominant viral quasispecies. We also demonstrated that sequence homology in the population gradually increased while quasispecies diversity decreased during continuous passages when an antiserum was absent. Our work suggests that the dominant quasispecies were most likely mutants that had lost their antigenic epitopes that recognized specific antisera; therefore, they were able to evade viral neutralizing activity.

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1 Conzelmann K K, Visser N, van Woensel P, *et al.* Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group. Virology, 1993, 193: 329–339
2 Snijder E J, Meulenberg J J M. The molecular biology of Arteriviruses. J Gen Virol, 1998, 79: 961–979
3 Key K F, Haqshenas G, Guenette D K, *et al.* Genetic variation and phylogenetic analysis of the *ORF5* gene of acute porcine reproductive and respiratory syndrome virus isolates. Vet Microbiol, 2001, 83: 249–263
4 Tian K, Yu X, Zhao T, *et al*. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS ONE, 2007, 2: e526
5 Li Y, Wang X, Bo K, *et al.* Emergence of a highly pathogenic porcine reproductive and respiratory syndrome virus in the mid-eastern region of China. Vet J, 2007, 174: 577–584
6 Tong G Z, Zhou Y J, Hao X F, *et al.* Emergence of the high pathogenic porcine reproductive and respiratory syndrome in China. Emerg Infect Dis, 2007, 13: 1434–1436
7 Wang Z, Cui Z. Evolution of *gp85* gene of subgroup J avian leukosis virus under the selective pressure of antibodies. Sci China Ser C-Life Sci, 2006, 49: 1–8
8 Lou B H, Zhu X T, Cui Z Z, *et al.* Mutations of the hemagglutinin gene of H9N2 subtype avian influenza viruses under selective pressure of antibody. Acta Microbiol Sin, 2009, 49: 955–959
9 Gong Y Y, Cui Z Z. Epitope variation in the Newcastle disease virus HN gene under antibody immune selective pressure in cell culture. Sci China Life Sci, 2011, 54: 474–479
10 Zhao P, Ma C T, Cui Z Z. Evolution of porcine reproductive and respiratory syndrome virus under antibody immune selective pressures. Sci Sin Vitae, 2010, 40: 952–962
11 Wei H, Tian K. Molecular mutations associated with the *in vitro* passage of virulent porcine reproductive and respiratory syndrome virus. Virus Genes, 2009, 38: 276–284
12 Liu H Q, Huang Y, Cheng J, *et al.* Genetic Mutations of the Hemagglutinin Gene of H9N2 subtype avian influenza viruses under the selective pressure of vaccination. Chin J Virol, 2002, 18: 149–150
13 Durate E A, Novella S C, Weaver S C, *et al.* RNA virus quasispecies: Significance for viral disease and epidemiology. Infect Agents Dis, 1994, 3: 201–204
14 Hwang S S, Boyle T J, Lyerly H K, *et al.* Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science, 1991, 253: 71–74
15 Shioda T, Levy L A, Chen M C. Small amino acid changes in the V3 hypervariable region of *gp120* can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type I. Proc Natl Acad Sci USA, 1992, 89: 9434–9438