Acta virologica **54**: 281 – 285, 2010 doi:10.4149/av_2010_04_281

Evidence for the adaptive evolution of ORF5 gene of Porcine reproductive and respiratory syndrome virus isolated in China

Z. XU, X. CHANG, S. XIAO, H. CHEN, R. ZHOU*

Division of Animal Infectious Disease, State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, P.R. China

Received February 8, 2010; accepted November 5, 2010

Summary. – Porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 gene encoding an envelope glycoprotein involved in humoral immunity is the most variable protein-coding gene of PRRSV. The present study aimed to identify potential selective pressures acting on the ORF5 gene of PRRSV isolates of North American type prevalent in China. The non-synonymous to synonymous rate ratio ω (d_N/d_S) was employed as a measure of selective pressure at the codon level. An overall ω of 0.45 indicated negative (purifying) selection as the major driving force operating on the ORF5 gene during adaptation of the virus to swine. Determination of ω values for individual amino acids sites revealed 8 positively selected sites, most of them situated in the N-terminal ectodomain, indicating their potential role in the binding of virus to the cellular receptors. Further, 75 negatively selected sites were identified in the rest of molecule, probably as a result of functional or immunological constraints. Determination of potential N-glycosylation sites revealed 7 sites, four of which coincided with the positively selected ones. These results indicated that a specific adaptive evolution has operated on the ORF5 gene of Chinese PRRSV isolates. It is hoped that the disclosed adaptive sites might help identify a candidate antigenic epitope for the use in vaccine against this serious swine disease.

Keywords: Porcine reproductive and respiratory syndrome virus; Chinese isolates; ORF5; adaptive evolution; positive selection; negative selection; glycosylation

Introduction

Porcine reproductive and respiratory syndrome is characterized by the severe reproductive failure in pregnant sows and respiratory disease in pigs of all ages (An *et al.*, 2007). The etiologic agent of this swine disease is PRRSV, which belongs to the family *Arteriviridae,* the order *Nidovirales* (Cavanagh, 1997; Snijder *et al.*, 2005). PRRSV is an enveloped virus containing positive-sense single-stranded RNA genome (Meulenberg *et al.*, 1993). The approximately 15 kb genome includes nine ORFs. ORF1a and ORF1b encode viral replicase polyproteins. ORF2a, ORF2b, and ORFs 3-7 encode the viral structural proteins GP2, E, GP3, GP4, GP5, M, and N, respectively (Meulenberg *et al.*, 1993; Stadejek *et al.*, 2002). Based on the sequence comparison of structural genes, PRRSV has been divided into two genotypes, the European type and North American type (Murtaugh *et al.*, 1995). Since the first identification of PRRSV in 1996, PRRSV isolates prevalent in China have been North American type according to the epidemiological data (Tian *et al.*, 2007; Zhou *et al.*, 2009).

The non-synonymous/synonymous substitution rate ratio ($\omega = d_N/d_S$) is a powerful measure of selective pressure at the codon level and $\omega = 1$ or $\omega < 1$ or $\omega > 1$ indicate neutral evolution, purifying (negative) selection, and diversifying (positive) selection, respectively (Yang *et al.*, 2000; Yang, 2000). Codon-substitution models that allow for the heterogeneous ω values among amino acid sites are widely used to study the mechanisms of adaptive evolution on the protein-

*Corresponding author. E-mail: rzhou@mail.hzau.edu.cn; fax: +86-27-87282608.

Abbreviations: d_N = the numbers of non-synonymous substitutions per site; d_S = the numbers of synonymous substitutions per site; GP5 = ORF5 glycoprotein; PRRSV = Porcine reproductive and respiratory syndrome virus; $\omega = d_N/d_S$

coding genes and to detect putative sites undergoing positive selection (Nielsen and Yang, 1998; Yang and Bielawski, 2000; Tang and Zhang, 2007; Vijaykrishna *et al.*, 2008). The evidence for positive selection operating on the virus genes is helpful to infer and identify molecular determinants of virulence or antigenicity without any prior knowledge of the pathogenic and immunologic mechanism (Tang *et al.*, 2008; Baillie *et al.*, 2008).

The ORF5 protein (GP5) is the major glycosylated envelope protein present on the virion surface that forms a disulfide-linked heterodimer with ORF6 protein (Mardassi *et al.*, 1996; Dea *et al.*, 2000). Three potential N-linked glycosylation sites involved in PRRSV immune evasion and persistence are located in the ectodomain comprising the first 40 amino acid residues of the mature GP5 protein (Mardassi *et al.*, 1996; Ansari *et al.*, 2006). Neutralizing antibodies induced by GP5 play an important role in the protection against PRRSV infection (Ostrowski *et al.*, 2002). Two antigenic epitopes have been identified in the ectodomain correlating with the recognition of PRRSV-specific antibodies (Ostrowski *et al.*, 2002). In addition, it is supposed that the ORF5 gene has the highest genetic polymorphism under constant selective pressure even within the same genotype (Kapur *et al.*, 1996; Li *et al.*, 2009). The replacement of specific amino acids undergoing diversifying selection may alter the properties of antigenic determinants and glycosylation sites hindering or enhancing the clearance of viruses *in vivo*. Thus, understanding the mechanism of the adaptive protein evolution on ORF5 is beneficial to the detection of crucial sites that enable viruses to escape the host immune system. Moreover, it could help to develop a candidate vaccine and drug against the PRRSV.

In the present study, we investigated the characteristics of the adaptive evolution on the ORF5 gene of the North American type PRRSV isolates originating from China.

Materials and Methods

Sequence data set and alignment. The ORF5 nucleotide sequences (600 bp) and corresponding amino acid sequences of PRRSV isolated in China were retrieved from the GenBank database (http://www.ncib.nlm.nih.gov) in November 2009. The sequences were manually checked and those of the European type isolates were excluded. Sequence alignment was performed using ClustalW algorithm (Li, 2003) with default parameters implemented in MEGA 4 (Kumar *et al.*, 2008). The aligned amino acid sequences were then converted to the coding nucleotide sequence alignment. The sequences that have more than 99% sequence identity were removed. We then tested whether recombination events existed in our data set by using the recombination detection program version 3 (Martin and Rybicki, 2000). In addition, nucleotide substitution saturation was tested for the alignment using DAMBE (Xia and Xie, 2001). The final data set consisted of 85 ORF5 gene sequences. The alignment is available upon request from the authors.

Nucleotide substitution model. Selection of the nucleotide substitution model that was optional for the phylogenetic inference of PRRSV ORF5 gene was conducted with program jModeltest (Posada, 2008). The nucleotide substitution model TPM2uf + I + Γ was identified as the best for fitting to ORF5 alignment. Based on the above substitution model, a phylogenetic tree was built using a maximum likelihood approach implemented in PhyML 3.0 (Guindon and Gascuel, 2003). The generated maximum likelihood tree was used for the subsequent selection analyses.

Detection of positively/negatively selected sites. To detect selective pressure acting on ORF5, synonymous and non-synonymous substitution rates were estimated site-by-site using the codeml program in PAML 4.2b package (Yang, 2007). According to the topology of the resulting maximum likelihood tree, five site-specific models that allow variable non-synonymous (d_N) and synonymous (d_S) rate ratios ($\omega = d_N/d_S$) among codons were applied to analyze our data set: M0 (one-ratio), M1 (NearlyNeutral), M2 (PositiveSelection), M7 (beta), and M8 (beta & ω). Model M0 evaluated the same ω value for all branches and sites in the phylogeny. Null hypothesis models M1 and M7 were nested with the alternative selection models M2 and M8. The latter two models separately added an extra site class for a fraction of positively selected sites with $\omega > 1$; whereas models M1 and M7 only allowed site classes with ω varying between 0 and 1 (Yang *et al.*, 2000; Wong *et al.*, 2004). The likelihood ratio tests (LRTs) were carried out to infer the occurrence of sites evolving under positive selection, comparing M1 against M2, and M7 against M8. In order to infer sites under negative selection on ORF5 and simultaneously evaluate the accuracy of those positively selected sites detected by PAML, our data set was further analyzed using the fixed effects likelihood (FEL) method (Kosakovsky and Frost, 2005a) via the DataMonkey website (Kosakovsky and Frost, 2005b).

Prediction of glycosylation sites. Putative N-linked glycosylation sites were predicted using a glycosylation analysis algorithm (Zhang *et al.*, 2004) implemented in the web server (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html).

Results and Discussion

There was no evidence for homologous recombination detected in our data set. Moreover, the result of transitions and transversions versus the genetic distance indicated that the ORF5 sequences did not experience substitution saturation and were fit to the subsequent phylogenetic analysis (Xia, 2000).

The maximum likelihood estimate of the average non-synonymous to synonymous substitution rate ratio was 0.45 over all sites in the ORF5 sequences indicating that purifying selection has been the major driving force for the evolutionary process of PRRSV ORF5 gene during adaptation to the swine. Based on the likelihood ratio test statistic for comparison of two pairs of nested models with the χ^2_2 distribution, null models M1 and M7 were significantly rejected in favor of the selection models M2 and M8 ($P < 0.001$), respectively. Thus, we confirmed that positively

Table 1. Positively selected amino acid sites in the ORF5 gene of Chinese PRRSV isolates

Model	Positively selected sites
M2: positive selection	32 33 34 35 58 59 102
M8: beta & $\omega > 1$	13 32 33 34 35 58 59 102

Amino acid site numbering is based on the reference sequence Acc.No. AY032626 of PRRSV CH-1a isolate.

selective force was indeed operating on the specific sites in the ORF5 gene of North American type PRRSV isolates from China. Model M8 identified all of the positively selected sites (32 to 35, 58, 59, and 102) detected by M2 and an additional site 13 (Table 1). All these adaptive sites except one (site 59) were also identified by the fixed effects models (Kosakovsky and Frost, 2005a). The corresponding selection profile was illustrated in Fig. 1. Meanwhile, 75 negatively selected sites (37.5%) were detected at the 0.1 significance level. As the amino acid substitutions at these sites undergoing negative selection are likely to be intolerable for the viral infectivity (Suzuki, 2004). They may become useful prime targets for the development of vaccines and antiviral drugs against PRRSV infections.

Glycosylation modification may mask important antigenic epitopes of the viral surface proteins. However, it is a primary mechanism for the enveloped viruses to escape or minimize the neutralizing immune response (Ansari *et al.*, 2006; Vigerust and Shepherd, 2007). Glycosylated sites in the N-terminal ectodomain of the ORF5 protein were thought to be able to cause a poor recognition of this main viral antigen by the swine antibodies (Ansari *et al.*, 2006). According to the glycosylation analysis, seven potential N-linked glycosylation sites (30, 32 to 35, 44, and 51) were predicted and four (sites 32, 33, 34, and 35) of them were found to be positively selected sites and adjacent to the both mapped antigenic epitopes (Ostrowski *et al.*, 2002). These glycosylation patterns were supported by the experimental evidence for PRRSV ORF5 protein (Dea *et al.*, 2000). The positively selected site 33, an N-linked glycosylation site was observed to have an obvious exchange between two polar amino acids. The corresponding mutation N33S may suppress the glycan-shielding effect and induce the high levels of neutralizing antibodies against PRRSV (Jiang *et al.*, 2007). It has been pointed out that amino acid sites under positive immune selection are strongly associated with N-linked glycosylation (Choisy *et al.*, 2004).

Interestingly, the intense signal for the positive selection was concentrated in the N-terminus represented by the first 35 residues of ORF5 protein, whereas the C-terminal end was mainly subject to the negative selective pressure, perhaps due to the functional or immunological constrains (Fig. 1). Six positively selected sites (32 to 35, 58, and 59) were located in the N-terminal ectodomain and extra site 13 in the signal peptide region. Previous reports (Dea *et al.*, 2000; Ostrowski *et al.*, 2002; Ansari *et al.*, 2006) showed that the N-terminal part of the ORF5 protein was involved in the receptor recognition and neutralization in the infection process. Notably, the positively selected site 102 was found to fall in the other ectodomain comprising a residue 89–109

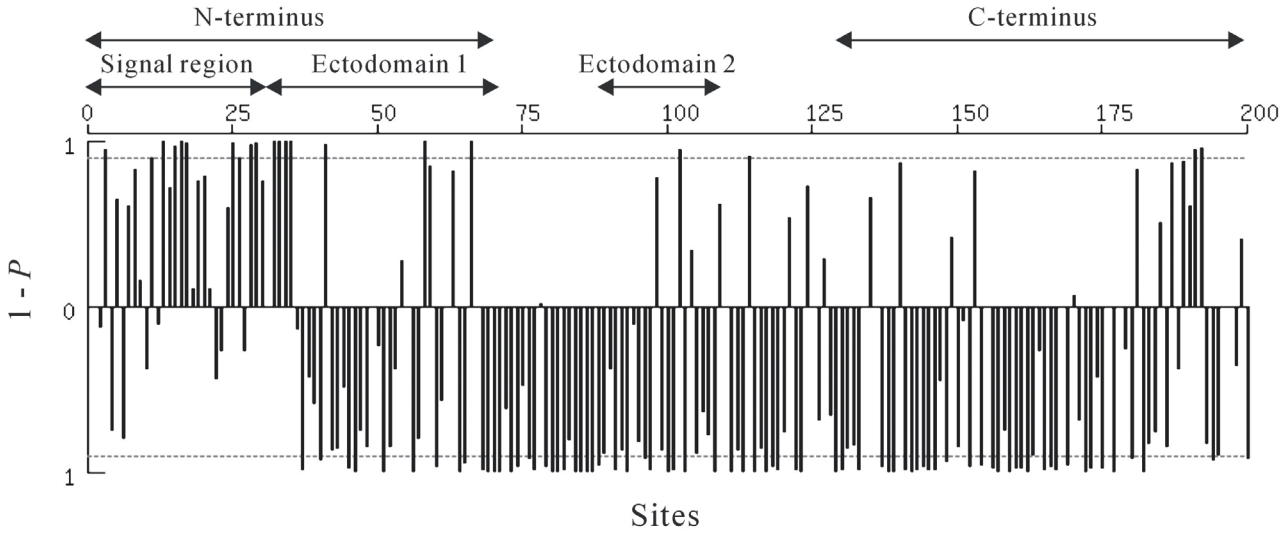

Fig. 1

Selection profile of the ORF5 coding region of North American type PRRSV isolates from China

The abscissa represents the amino acid positions. The ordinate represents the value of (1–*P*) for each position, which is indicated above and below the abscissa when $d_N/d_S > 1$ and $d_N/d_S < 1$, respectively. The dashed lines represent the significant threshold set at 0.1.

segment of the ORF5 protein, which might be involved in the virus antigenicity (Stadejek *et al.*, 2002; Pesch *et al.*, 2005). Therefore, those positively selected sites located in the two ectodomains possibly contributed to the interaction between PRRSV ORF5 protein and host receptors and consequently evolved rapidly to escape the host immune system. In addition, the positively selected site R13 might be associated with the virulence as a result of R13S mutation observed between the virulent VR-2332 isolate and RespPRRS/Repro vaccine strain (Allende *et al.*, 2000).

In comparison to the previous results obtained from 140 ORF5 sequences of North American PRRSV isolates without considering diverse geographical locations of isolation (Hanada *et al.*, 2005), our study demonstrated that PRRSV isolated from China had some special evolutionary patterns of natural selection acting on ORF5 during adaptation to the swine. Several sites (33, 34, 58, 59, and 102) were firstly identified to experience significant positive selection hinting their potential role in association with the functional features. The results herein can provide the important clues for thoughts about the candidate antigenic epitopes important for immunization, because amino acid substitutions at the adaptive sites could result in the ineffective receptor binding-sites and help PRRSV to escape the immune system (Hanada *et al.*, 2005; Suzuki, 2006). The generations of these adaptive sites may be ascribed to the host immunological pressure probably due to the increased vaccination against PRRSV in recent years (Hu *et al.*, 2009).

In conclusion, by using the maximum likelihood estimation we found evidence for the adaptive evolution on the envelope glycoprotein ORF5 gene of Chinese PRRSV isolates and identified eight sites subjected to the strong positive selective pressure. It is necessary to experimentally inspect, whether amino acid replacements in these positively selected sites potentially affect the alteration of antigenic epitopes and influence the glycosylation effects. These results provide the valuable information for further examination of the mechanism of PRRSV immune evasion and for the development of vaccine and antiviral drug for the control and prevention of this disease in China.

Acknowledgment. This work was supported by the grant 2006BAD06A01 from the National Scientific & Technical Supporting Program of China.

Reference

Allende R, Kutish GF, Laegreid W, Lu Z, Lewis TL, Rock DL, Friesen J, Galeota JA, Doster AR, Osorio FA (2000): Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. Arch. Virol. 145, 1149–1161. doi:10.1007/s007050070115

An TQ, Zhou YJ, Liu GQ, Tian ZJ, Li J, Qiu HJ, Tong GZ (2007): Genetic diversity and phylogenetic analysis of glycoprotein 5 of PRRSV isolates in mainland China from 1996 to 2006: coexistence of two NA-subgenotypes with great diversity. Vet. Microbiol. 123, 43–52. doi:10.1016/j.vetmic.2007.02.025

Ansari IH, Kwon B, Osorio FA, Pattnaik AK (2006): Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J. Virol. 80, 3994–4004. doi:10.1128/JVI.80.8.3994-4004.2006

Baillie GJ, Kolokotronis SO, Waltari E, Maffei JG, Kramer LD, Perkins SL (2008): Phylogenetic and evolutionary analyses of St. Louis encephalitis virus genomes. Mol. Phylogenet. Evol. 47, 717–728. doi:10.1016/j.ympev.2008.02.015

Cavanagh D (1997): Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142, 629–633.

Choisy M, Woelk CH, Guégan JF, Robertson DL (2004): Comparative study of adaptive molecular evolution in different human immunodeficiency virus groups and subtypes. J. Virol. 78, 1962–1970. doi:10.1128/JVI.78.4.1962-1970.2004

Dea S, Gagnon CA, Mardassi H, Pirzadeh B, Rogan D (2000): Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch. Virol. 145, 659–688. doi:10.1007/s007050050662

Guindon S, Gascuel O (2003): A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704. doi:10.1080/10635150390235520

Hanada K, Suzuki Y, Nakane T, Hirose O, Gojobori T (2005): The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol. Biol. Evol. 22, 1024-1031. doi:10.1093/molbev/msi089

Hu H, Li X, Zhang Z, Shuai J, Chen N, Liu G, Fang W (2009): Porcine reproductive and respiratory syndrome viruses predominant in southeastern China from 2004 to 2007 were from a common source and underwent further divergence. Arch. Virol. 154, 391–398. doi:10.1007/s00705-009-0316-x

Jiang W, Jiang P, Wang X, Li Y, Wang X, Du Y (2007): Influence of porcine reproductive and respiratory syndrome virus GP5 glycoprotein N-linked glycans on immune responses in mice. Virus Genes 35, 663–671. doi:10.1007/s11262-007-0131-y

Kapur V, Elam MR, Pawlovich TM, Murtaugh MP (1996): Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. 77, 1271–1276. doi:10.1099/0022-1317-77-6-1271

Kosakovsky Pond SL, Frost SD (2005a): Not so different after all: a comparison of methods for detecting amino acid sites under selection. Mol. Biol. Evol. 22, 1208–1222. doi:10.1093/molbev/msi105

Kosakovsky Pond SL, Frost SD (2005b): Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 21, 2531–2533. doi:10.1093/bioinformatics/bti320

Kumar S, Nei M, Dudley J, Tamura K (2008): MEGA: a biologist-centric software for evolutionary analysis of DNA

and protein sequences. Brief Bioinform. 9, 299–306. doi:10.1093/bib/bbn017

Li KB (2003): ClustalW-MPI: ClustalW analysis using distributed and parallel computing. Bioinformatics 19, 1585–1586. doi:10.1093/bioinformatics/btg192

Li Y, Wang X, Jiang P, Wang X, Chen W, Wang X, Wang K (2009): Genetic variation analysis of porcine reproductive and respiratory syndrome virus isolated in China from 2002 to 2007 based on ORF5. Vet. Microbiol. 138, 150–155. doi:10.1016/j.vetmic.2009.03.001

Mardassi H, Massie B, Dea S (1996): Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology 221, 98–112. doi:10.1006/viro.1996.0356

Martin D, Rybicki E (2000): RDP: detection of recombination amongst aligned sequences. Bioinformatics 16, 562–563. doi:10.1093/bioinformatics/16.6.562

Meulenberg JJ, Hulst MM, de Meijer EJ, Moonen PL, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJ (1993): Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192, 62–72. doi:10.1006/viro.1993.1008

Murtaugh MP, Elam MR, Kakach LT (1995): Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. Arch. Virol. 140, 1451–1460. doi:10.1007/BF01322671

Nielsen R, Yang Z (1998): Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. Genetics 148, 929–936.

Ostrowski M, Galeota JA, Jar AM, Platt KB, Osorio FA, Lopez OJ (2002): Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76, 4241–4250. doi:10.1128/JVI.76.9.4241-4250.2002

Pesch S, Meyer C, Ohlinger VF (2005): New insights into the genetic diversity of European porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Microbiol. 107, 31–48. doi:10.1016/j.vetmic.2005.01.028

Posada D (2008): jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256. doi:10.1093/molbev/msn083

Snijder EJ, Brinton MA, Faaberg KS., Godeny EK, Gorbalenya AE, MacLachlan NJ, Mengeling WL Plagemann PGW (2005): Family Arteriviridae. In Fauqet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (Eds): Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. Academic Press, Elsevier-London, pp. 965–974.

Stadejek T, Stankevicius A, Storgaard T, Oleksiewicz MB, Belák S, Drew TW, Pejsak Z (2002): Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. J. Gen. Virol. 83, 1861–1873.

Suzuki Y (2004): Negative selection on neutralization epitopes of poliovirus surface proteins: implications for prediction of candidate epitopes for immunization. Gene 328, 127–133.

Suzuki Y (2006): Natural selection on the influenza virus genome. Mol. Biol. Evol. 23, 1902–1911. doi:10.1093/molbev/msl050

Tang F, Pan Z, Zhang C (2008): The selection pressure analysis of classical swine fever virus envelope protein genes Erns and E2. Virus Res. 131, 132–135. doi:10.1016/j.virusres.2007.08.015

Tang F, Zhang C (2007): Evidence for positive selection on the E2 gene of bovine viral diarrhoea virus type 1. Virus Genes 35, 629–634. doi:10.1007/s11262-007-0122-z

Tian K, Yu X, Zhao T, Feng Y, Cao Z, Wang C, Hu Y, Chen X, Hu D, Tian X, Liu D, Zhang S, Deng X, Ding Y, Yang L, Zhang Y, Xiao H, Qiao M, Wang B, Hou L, Wang X, Yang X, Kang L, Sun M, Jin P, Wang S, Kitamura Y, Yan J, Gao GF (2007): Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS One 2, e526. doi:10.1371/journal.pone.0000526Vigerust DJ, Shepherd VL (2007): Virus glycosylation: role in virulence and immune interactions. *Trends Microbiol.* **15**, 211–218.

Vigerust DJ, Shepherd VL (2007): Virus glycosylation: role in virulence and immune interactions. Trends Microbiol. 15, 211–218. doi:10.1016/j.tim.2007.03.003

Vijaykrishna D, Bahl J, Riley S, Duan L, Zhang JX, Chen H, Peiris JS, Smith GJ, Guan Y (2008): Evolutionary dynamics and emergence of panzootic H5N1 influenza viruses. PLoS Pathog. 4, e1000161. doi:10.1371/journal.ppat.1000161

Wong WS, Yang Z, Goldman N, Nielsen R (2004): Accuracy and power of statistical methods for detecting adaptive evolution in protein coding sequences and for identifying positively selected sites. Genetics 168, 1041–1051. doi:10.1534/genetics.104.031153

Xia X (2000): Data analysis in molecular biology and evolution. Kluwer Academic Publishers, Boston, Massachusetts, pp. 115–124.

Xia X, Xie Z (2001): DAMBE: software package for data analysis in molecular biology and evolution. J. Hered. 92, 371–373. doi:10.1093/jhered/92.4.371

Yang Z (2000): Maximum likelihood estimation on large phylogenies and analysis of adaptive evolution in human influenza virus A. J. Mol. Evol. 5, 423–432.

Yang Z (2007): PAML 4: phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 24, 1586–1591. doi:10.1093/molbev/msm088

Yang Z, Bielawski JP (2000): Statistical methods for detecting molecular adaptation. Trends Ecol. Evol. 15, 496–503. doi:10.1016/S0169-5347(00)01994-7

Yang Z, Nielsen R, Goldman N, Pedersen AM (2000): Codon-substitution models for heterogeneous selection pressure at amino acid sites. Genetics 155, 431–449.

Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, Korber B (2004): Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology 14, 1229–1246. doi:10.1093/glycob/cwh106

Zhou YJ, Yu H, Tian ZJ, Li GX, Hao XF, Yan LP, Peng JM, An TQ, Xu AT, Wang YX, Wei TC, Zhang SR, Cai XH, Feng L, Li X, Zhang GH, Zhou LJ, Tong GZ (2009): Genetic diversity of the ORF5 gene of porcine reproductive and respiratory syndrome virus isolates in China from 2006 to 2008. Virus Res. 144, 136–144. doi:10.1016/j.virusres.2009.04.013