Evolutionary Diversity of Suid Herpesvirus 1 Based on UI44 Partial Sequences

Antônio Augusto Fonseca Jr.^a Marcelo Fernandes Camargos^a
Aline Aparecida Silva Barbosa^a Valdênia Lopes Silva Gonçalves^a
Marcos Bryan Heinemann^b Jenner K. Pimenta dos Reis^c

^aLaboratório de Biologia Molecular, Lanagro/MG, Pedro Leopoldo, and ^bDepartamento de Medicina Veterinária Preventiva e Saúde Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, ^cDepartamento de Medicina Veterinária Preventiva da Escola de Veterinária da Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

Key Words

Suid herpesvirus 1 · Pseudorabies · Phylogeny

Abstract

Objective: The aim of this study was to use partial UI44 sequences (glycoprotein C) of Suid herpesvirus 1 to examine the evolution and dynamics of the virus in different periods and hosts. **Methods:** Phylogenetic trees were constructed using the software MrBayes after analysis in the software jModelTest to evaluate the best phylogenetic models. The software SplitsTree 4.0 was used to create phylogenetic networks, and the BEAST program was used to generate data on phylogeography. Replication kinetics and serum neutralization tests were applied to tree strains from different phylogenetic groups. **Results:** UI44 sequences derived from domestic swine and wild swine clustered in different clades and had different selective pressures depending on the host. We found no differences in replication kinetics and serum neutralization tests in the strains tested. Data show that the evolution of herpesviruses is complex, and different genetic

groups may be evolving at different rates. UI44 is an important marker for molecular evolution and epidemiology studies, but it is not useful for biological information.

© 2016 S. Karger AG, Basel

Introduction

Suid herpesvirus 1 (SuHV-1) is the causative agent of pseudorabies, a disease of great impact in the swine industry. SuHV-1 (Alphaherpesvirinae) is a slowly evolving double-stranded DNA virus [1]. The main hosts of SuHV-1 are pigs (*Sus scrofa*). Domestic pigs are susceptible to infection with clinical signs that vary according with age. There is a high lethality rate in young animals due to neurologic signs; however, in older individuals, mortality is reduced and the major clinical signs are respiratory and reproductive [1]. Due to its ability to produce a latent infection, SuHV-1 can remain in the herd undetected, generating new outbreaks of disease even after long periods without clinical manifestations [2].

The intensive combat against pseudorabies in the last 20 years has led to the eradication of the disease in commercial herds in various countries in North America and Europe [3]. Other countries, like Brazil, have advanced eradication programs with very few or no reports of the disease in the last decade [4]. As a consequence of the advancement of eradication programs, SuHV-1 has spread to wild swine (WS), including wild boar or feral pigs. Infection has been detected in these animals in many countries in America and Europe through either molecular or serological methods [5–8].

Studies on the evolution of SuHV-1 have compared virulence and genetic characteristics based on field isolates from several hosts and years. Viruses isolated from domestic pigs in different periods showed different replication and infection rates [9]. Experimental infection of DS with the SuHV-1 strain from WS was not able to reproduce the disease, demonstrating the low virulence of these isolates [10]. The genetic characteristics also varied according to the period of isolation or host [11].

Initial studies of the genetic variation of SuHV-1 were based on restriction fragment length polymorphism with *Bam*HI, demonstrating the presence of four genomic types [12, 13]. More recent studies have used sequencing, particularly gene *UL44*, which codes for glycoprotein C [4–6, 14]. The problem with these analyses is that they only focus on conventional phylogenetic analysis by reconstructing phylogenetic trees, and they do not evaluate genetic distances or use other methods to verify conflicting signals within the phylogenetic trees.

The advantage of sequencing is that it allows several types of analysis, such as phylogeography and phylodynamics, which is the study of how viral mutations affect and are affected by factors such as host immunity and the dynamic transmission of epidemics. Therefore, the data generated in all these publications can be used to access more information about SuHV-1 [14]. SuHV-1 can be divided into at least five genetic groups using phylogenetic analysis based on partial *UL44* sequences [15]. These groups have different evolution rates and separate viruses isolated from DS and WS; they also show that viruses isolated in Asia are more genetically diverse than viruses isolated in Europe and America. Phylogenetic analysis using other genes also shows a clear separation between samples isolated in Western and Eastern countries and shows a rapid dispersion of SuHV-1 in China [16].

The aim of this study was to use partial sequences of SuHV-1 genes to examine the evolution and dynamics in different periods, places, or hosts. To our knowledge, we used phylogenetic tools that have not been used in other

publications, like networks and phylogeography, to better understand the genetic profiles of viruses isolated in DS and WS.

Methods

Genetic Sequences

215 partial *UL44* nucleotide sequences of SuHV-1 were collected from GenBank with the largest possible amount of information available on host, year, and country of isolation [4–6, 8, 17–23]. The sequences were identified using the name of the isolate in GenBank followed by a three-letter code to identify the country of origin: Germany (GER), Argentina (ARG), Austria (AUS), Brazil (BRA), China (CHI), Croatia (CRO), Greece (GRE), South Korea (SKO), Slovakia (SLK), Spain (ESP), United States (USA), Japan (JAP), Hungary (HUN), Italy (ITA), Northern Ireland (NIR), Malaysia (MAL), or Sweden (SWE). We also included sequences derived from vaccine strains like Bartha.

Phylogenetic Analysis

The evolutionary models were selected with the aid of the computer program jModelTest 2 [24] and based on the procedures described by Fonseca et al. [15]. Phylogenetic trees were constructed using model GTR Γ 4+I in MrBayes.

The mean (relative) evolutionary rate for each isolate was estimated under the Jones-Taylor-Thornton (1992) model (+G) [1]. A discrete Gamma (+G) distribution was used to model evolutionary rate differences among sites (5 categories). The maximum likelihood estimate of the gamma shape parameter was 0.0500. The maximum log likelihood for this computation was –757.914. The analysis involved 25 amino acid sequences. The coding data was translated assuming a standard genetic code table. All positions containing gaps and missing data were eliminated. There were a total of 214 positions in the final data set. Evolutionary analyses were conducted in MEGA6 [25].

The software SplitsTree 4.0 was used for evaluation of recombination and conflicting positions in the phylogeny [26]. DnaSP [27] and Network [28] were used for phylogenetic network analysis of partial *UL44* sequences.

Phylogeography

Phylogeography tests were performed with partial sequences of *UL44* from three separate groups depending on origin: domestic swine (DS) of Brazil and the United States and WS of Europe. After selecting the groups, the data were verified as to the precise isolation of each strain. Phylogeographic analyses were then performed with a group of Brazilian sequences derived from outbreaks in farms in the South and Southeast that occurred between 1983 and 2003 [4]. The sequence analysis of American samples was performed according to the data described by Goldberg et al. [14], with samples isolated from pigs and also from terminal hosts of the virus like dogs, cows, and sheep. The last group of samples studied was European WS [6].

Analyses were performed in the program BEAST [29] with the following parameters: GTR + I Γ 4 with the 5×10^9 MCMC sampling every 1,000 states with adaptations for continuous and discrete data according to Lemey et al. [30]. The results were submitted to the Spread program for discrete tree, continuous tree and Time Slicer analysis [31].

Fig. 2. Mean (relative) evolutionary rates are shown for each site. These rates are scaled such that the average evolutionary rate across all sites is 1. This indicates that sites showing a rate <1 are evolving slower than average and those with a rate >1 are evolving faster than average.

Table 1. Genetic distance between groups and within groups

	Between groups					Within groups
	A	B	C	D	E	
A						0.000
B	0.021					0.001
C	0.019	0.025				0.007
D	0.019	0.019	0.019			0.003
E	0.033	0.037	0.047	0.049		0.012
F	0.014	0.015	0.016	0.016	0.042	0.007

Fonseca et al. [15]. There is evidence of subgroup formation in groups B, C, D, and E, but samples from group A are stable with little genetic distance (table 1). Most of the genetic distance within group C was generated by the presence of samples from different countries. This group can be divided into subgroups like C1 (from Germany and Austria), C2 (from Hungary), and C3 (from Slovakia and vaccine strain Bartha) with genetic distances 0.004, 0.005, and 0.005, respectively. Group C has the highest

mean relative rates for the polymorphic sites, but has fewer sites than groups E and D (fig. 2).

We found a total of 53 genetic profiles for *U144* when the analysis was performed with all sequences, including two specific profiles for vaccines. When populations were divided, we found 10 genetic profiles for isolates from WS, with an overall genetic distance of 0.013, and 19 genetic profiles for DS, with an overall genetic distance of 0.018. Most mutations found involved codon deletions/insertions.

There were four conserved regions (211–259, 346–430, 472–503, and 569–618), and they were the same when WS and DS isolates were compared. Codon usage bias indicated only a discrete difference between these two groups of isolates in the use of codons for serine and valine.

There were differences among genetic profiles from SuHV-1 isolated from WS and DS. The WS sequences analyzed in this study had 31 polymorphic sites (with 31 mutations, 7 singleton variable sites, and 24 parsimony informative sites), with 6 of these monomorphic in DS.

Fig. 3. Phylogenetic network constructed from partial *U144* sequences of SuHV-1 isolates using the software Network. Red (color in online version only) represents sequences from strains isolated in Asia, green from strains isolated in South America, yellow from strains isolated in USA, light blue from strains isolated from

DS in Europe, and dark blue from strains isolated from WS in Europe. The center of the network is formed by European sequences from Germany, Austria, and Italy. These sequences belong to group D in the phylogenetic trees.

Among the DS sequences, there were 49 polymorphic sites (51 mutations, 24 singleton variable sites, and 25 parsimony informative sites), with 26 of these monomorphic in WS. The two groups shared 24 mutations.

Phylogenetic construction using the software Network revealed that when all sequences were used, those derived from Europe were concentrated at the center, from which the branches extend to the formation of genotypes from SuHV-1 isolated from DS, but the presence of median vectors indicate that there is still missing information in the base of the network (fig. 3). Again, the Asian samples exhibit the same behavior as in the phylogenetic trees.

Phylogeography

DS sequences from Brazil showed no genetic differentiation, so it was not possible to perform an accurate phylogeographic analysis. Sequences from the US had a more variable pattern. The results with the discrete data demonstrate a rapid change of the genetic variation of SuHV-1 in very close locations (fig. 4). Continuous data show that genetic variation increases first in the North and then West. These latest results showed the locations with out-

breaks in pigs in important points in the geographical distribution.

The data generated with the phylogeographic analysis using WS sequences showed a consistent distribution pattern with the main points of genetic variability in Hungary and Slovakia, with posterior distribution to Eastern Germany and then to the Western portions of the continent like Northern France and Spain (fig. 4). The SuHV-1 varies little between these locations, remaining in homogeneous groups for long periods. The analysis by Time Slicer proposes the location of the root of the phylogenetic tree in Switzerland, but with low posterior probabilities. The same occurs for the positioning of a node in the center of France (fig. 5).

Serological and Replication Characteristics

All three isolates had the same titer variation in the different cell lines used. ShopeHUN had titers $10^{5.66}$ TCID₅₀/50 μ l in PK15, $10^{6.5}$ TCID₅₀/50 μ l in MDBK, and $10^{5.60}$ TCID₅₀/50 μ l in RK13. 3303BRA had titers $10^{3.57}$ TCID₅₀/50 μ l in PK15, $10^{3.5}$ TCID₅₀/50 μ l in MDBK, and $10^{3.1}$ TCID₅₀/50 μ l in RK13. 3338BRA had titers $10^{5.80}$ TCID₅₀/50 μ l in PK15, $10^{6.5}$ TCID₅₀/50 μ l in MDBK, and

Fig. 4. Discrete data analysis incorporated into the phylogenetic tree for samples from outbreaks of pseudorabies occurring in Illinois, United States. The color gradient of each line indicates the age of migration. IL1 to IL4, IL7, IL8, and IL12 indicate locations where SuHV-1 was isolated from pigs.

$10^{5.6}$ TCID₅₀/50 μ l in RK13. There were no significant variations in virus neutralization test as all results in the assay presented similar results with neutralization occurring in a dilution of 1:16 for ShopeHUN and 3338BRA and 1:32 for 3303BRA. Replication characteristics were similar for all isolates regardless of cell line (fig. 6). DNA from progeny virus was detected within 6 h with a rapid increase in 24 h.

Discussion

Pseudorabies eradication programs have restricted the number of outbreaks of the disease to the point of eradicating it from commercial herds of DS in many countries, but it still remains a global concern because of outbreaks in various locations such as Eastern Europe [16]. The prevalence of SuHV-1 in DS in these regions is not the sole problem because the virus persists in herds of wild

Fig. 5. Images generated from analysis by time slicer distribution of the SuHV-1 gene in wild boars in Europe. The circles indicate the relative time dispersion for the virus.

boar and feral pigs in various parts in the world, with high prevalence rates in certain regions [34].

SuHV-1 isolates are genetically gathered in five main groups distributed worldwide [15]. Among these, it is noteworthy that there are groups with sequences almost exclusively from SuHV-1 derived from wild boar, domestic pig, or Asian origin with a high degree of polymorphism. This data indicates that different strains of the virus are infecting these populations, a fact that has already

been suggested by results of characterization of isolates from WS [35].

No evidence has been found for positive selection of isolates from WS, but *UL44* sequences from DS are under strong positive selection. Genetic variability was even more pronounced when the two viral populations were compared separately. Even if positive selection was not detected in sequences from WS, the nucleotide substitution rate of *UL44* from these SuHV-1 isolates may be un-

Fig. 6. Graph demonstrating the kinetics of replication of three SuHV-1 strains Shope (group C, *Bam*HI genotype I), 3303BRA (group B, *Bam*HI genotype I), and 3338BRA (group D, *Bam*HI genotype II) in three different cell lines (MDBK, PK15, and RK13). The isolates showed similar kinetics of viral multiplication during the experiment with higher affinity for breeding in MDBK and PK15.

der the influence of other evolutionary forces such as genetic drift [36].

The phylogenetic networks formed by the *UL44* sequences confirm the division of SuHV-1 into at least five groups as described before [15] and show the conflicting signals that may create different phylogenetic trees according to the phylogenetic method used. Different number of samples, sequence size, and method for tree reconstruction may generate different results, making the comparison between studies difficult [8, 11, 14, 15].

Group D is located in the center of the network with WS sequences from Europe connecting other groups. This structure indicates the European origin of the SuHV-1 isolated in the West in the 20th and 21st centuries. This hypothesis is supported by other data, such as the presence of European sequences in all phylogenetic groups and the greatest genetic diversity in this continent. Among WS isolates, the sequences of Eastern Germany, Hungary, and Slovakia, even isolated in close regions and short intervals of time, are more genetically diverse than elsewhere in Europe, as was demonstrated by the division of group C into at least three subgroups. The high evolutionary rates in each sequence and the lack of positive selection described previously [15] shows that these viruses have been evolving by genetic drift, likely for a long time, as SuHV-1 can be highly stable for long periods of time. It should also be noted that most of the genetic diversity of the European SuHV-1 corresponds to the same region where the two major pig haplotypes were found [37, 38] and with the root calculated in phylogeographical analysis using sequences from virus related to WS outbreaks.

The genetic diversity of SuHV-1 decreases as it spreads westward in Europe. Samples from Spain, Northern France, and Western Germany had the same genetic profile for *UL44*. This is not a unique phenomenon seen in the genetics of SuHV-1. Brazilian isolates from outbreaks that occurred between 1983 and 2003 showed the same genetic profile [4]. This low diversity may be explained by the fact that herpesviruses evolve slowly [39]. SuHV-1 may have been introduced recently in these areas compared to the areas further east in Europe.

The Asian samples were divided genetically into two groups, one group that is exclusively formed by sequences derived from Asian isolates (group E) and a second that is distributed into other clades. This genetic difference is also shown in other genes and in phylogenetic networks constructed using partial *us8* sequences [16]. This exacerbated difference associated with the high genetic diversity of the group may be related to a rapid dispersion of SuHV-1 since its first recorded case in 1950s [22], and it could possibly be influenced by the fact that Asian pigs possess a more diverse genetic profile than Western pigs [38]. There is also evidence of genetic recombination between vaccine strains like Bartha, a characteristic that may alter the position of the strains in the phylogenetic tree [20].

The presence of Asian samples in groups A–D of the *UL44* phylogenetic tree may be associated with the worldwide distribution of pig breeds or with the procedure of using some Chinese breeds characterized by a high degree of fecundity to accelerate genetic progress [39].

Genetic differences in *UL44* do not seem to be related to other biological virus differences. The results for viral neutralization and titration and replication in different

cell lines showed no difference for two strains isolated in Brazil and for standard strain Shope. The same phenomenon has been previously reported for European strains isolated from WS and DS. Virus isolates showed no apparent in vitro differences in replication kinetics and sensitivity to interferon-induced antiviral status in the ST cell line and primary porcine cells established from kidney, lung, skin, and testicular cells [40]. Although there are differences in disease outcome and virus shedding between viruses isolated from DS and WS, there was no correlation between the position of the isolates in the phylogenetic tree and virulence even when tests were carried out in vivo [41]. It is possible that aspects of virulence are related to other markers in the genome and other biological characteristics such as viral and host microRNAs [42].

The results of this study demonstrate the coevolution of SuHV-1 and their hosts. The virus shows different evolution in clades of the phylogenetic trees. This difference can be seen between the sequences derived from feral pigs and domestic pigs and is more prominent when comparing Asian and Western isolates. The evolution of SuHV-1, a virus whose associated disease is already deeply influenced by host age, is strongly affected by genetics within *S. scrofa*. The immune system of pigs has substantial genetic variation [38] that is influenced by breed and age

[43], and may also have differentiated selection patterns as occurred in other genes [44]. All host genetic variability is important for viral evolution.

Moreover, our results demonstrate that SuHV-1, a slowly evolving virus, can have differences in genetic variability depending on the location or type of animal from which it is isolated. The virus evolves differently according to which host it infects because of the different selection pressures to which it is subjected. *UL44* is an important genetic marker for epidemiologic and evolutionary analysis, but it does have biological properties like replication and serum neutralization.

Acknowledgments

The authors wish to thank Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais for financial support of English proofreading and INCT-Pecuária, CNPq, and FAPEMIG for financial support of this work. J.K.P.d.R. and M.B.H. received fellowships from CNPq. We would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – CAPES – Brasil for financial support.

Disclosure Statement

The authors declare that they have no competing interests.

References

- 1 Pomeranz LE, Reynolds AE, Hengartner CJ: Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. *Microbiol Mol Biol Rev* 2005;69:462–500.
- 2 Romero CH, Meade PN, Homer BL, Shultz JE, Lollis G: Potential sites of virus latency associated with indigenous pseudorabies viruses in feral swine. *J Wildl Dis* 2003;39:567–575.
- 3 Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, ed 7. Paris, OIE, 2012.
- 4 Fonseca AA Jr, Camargos MF, de Oliveira AM, Ciacci-Zanella JR, Patrício MA, Braga AC, Cunha ES, D'Ambros RM, Heinemann MB, Leite RC, dos Reis JK: Molecular epidemiology of Brazilian pseudorabies viral isolates. *Vet Microbiol* 2010;141:238–245.
- 5 Hahn EC, Fadl-Alla B, Lichtensteiger CA: Variation of Aujeszky's disease viruses in wild swine in USA. *Vet Microbiol* 2010;143:45–51.
- 6 Müller T, Klupp BG, Freuling C, Hoffmann B, Mojczic M, Capua I, Palfi V, Toma B, Lutz W, Ruiz-Fon F, Gortázar C, Hlinak A, Schaarschmidt U, Zimmer K, Conraths FJ, Hahn EC, Mettenleiter TC: Characterization of pseudorabies virus of wild boar origin from Europe. *Epidemiol Infect* 2010;138:1590–1600.
- 7 Mahmoud HY, Suzuki K, Tsuji T, Yokoyama M, Shimajima M, Maeda K: Pseudorabies virus infection in wild boars in Japan. *J Vet Med Sci* 2011;73:1535–1537.
- 8 Paes RC, Fonseca AA Jr, Monteiro LA, Jardim GC, Piovezan U, Herrera HM, Mauro RA, Vieira-da-Motta O: Serological and molecular investigation of the prevalence of Aujeszky's disease in feral swine (*Sus scrofa*) in the subregions of the Pantanal wetland, Brazil. *Vet Microbiol* 2013;165:448–454.
- 9 Glorieux S, Favoreel HW, Meesen G, de Vos W, Van den Broeck W, Nauwynck HJ: Different replication characteristics of historical pseudorabies virus strains in porcine respiratory nasal mucosa explants. *Vet Microbiol* 2009;136:341–346.
- 10 Müller TF, Teuffert J, Zellmer R, Conraths FJ: Experimental infection of European wild boars and domestic pigs with pseudorabies viruses with differing virulence. *Am J Vet Res* 2001;62:252–258.
- 11 Müller T, Klupp B, Zellmer R, Teuffert J, Ziedler K, Possardt C, Mewes L, Dresenkamp B, Conraths FJ, Mettenleiter TC: Characterisation of pseudorabies virus isolated from wild boar (*Sus scrofa*). *Vet Rec* 1998;143:337–340.
- 12 Christensen LS: The population biology of suid herpesvirus 1. *APMIS Suppl* 1995;48:1–48.
- 13 Fonseca AA Jr, Magalhães CG, Sales EB, D'Ambros RM, Ciacci-Zanella J, Heinemann MB, Leite RC, Dos Reis JK: Genotyping of the pseudorabies virus by multiplex PCR followed by restriction enzyme analysis. *ISRN Microbiol* 2011;2011:458294.
- 14 Goldberg TL, Weigel RM, Hahn EC, Scherba G: Comparative utility of restriction fragment length polymorphism analysis and gene sequencing to the molecular epidemiological investigation of a viral outbreak. *Epidemiol Infect* 2001;126:415–424.
- 15 Fonseca AA Jr, Camargos MF, Sales ML, Heinemann MB, Leite RC, Reis JK: Pseudorabies virus can be classified into five genotypes using partial sequences of UL44. *Braz J Microbiol* 2012;43:1632–1640.
- 16 Fonseca Jr AA, Sales EB, Heinemann MB, Reis JK: Molecular phylogeny of suid herpesvirus 1. *ISRN Virol* 2014;2014:1–7.

- 17 Sozzi E, Moreno A, Lelli D, Cinotti S, Alborelli GL, Nigrelli A, Luppi A, Bresola M, Cattella A, Cordioli P: Genomic characterization of pseudorabies virus strains isolated in Italy. *Transbound Emerg Dis* 2014;61:334–340.
- 18 Ishikawa K, Tsutsui M, Taguchi K, Saitoh A, Muramatsu M: Sequence variation of the gC gene among pseudorabies virus strains. *Vet Microbiol* 1996;49:267–272.
- 19 Serena MS, Metz GE, Mórtoła EC, Echeverría MG: Phylogenetic analysis of Suid herpesvirus 1 isolates from Argentina. *Vet Microbiol* 2011;154:78–85.
- 20 Steinrigl A, Revilla-Fernández S, Kolodziejek J, Wodak E, Bagó Z, Nowotny N, Schmoll F, Köfer J: Detection and molecular characterization of Suid herpesvirus type 1 in Austrian wild boar and hunting dogs. *Vet Microbiol* 2012;157:276–284.
- 21 Luo Y, Li N, Cong X, Wang CH, Du M, Li L, Zhao B, Yuan J, Liu DD, Li S, Li Y, Sun Y, Qiu HJ: Pathogenicity and genomic characterization of a pseudorabies virus variant isolated from Bartha-K61-vaccinated swine population in China. *Vet Microbiol* 2014;174:107–115.
- 22 Ye C, Guo JC, Gao JC, Wang TY, Zhao K, Chang XB, Wang Q, Peng JM, Tian ZJ, Cai XH, Tong GZ, An TQ: Genomic analyses reveal that partial sequence of an earlier pseudorabies virus in China is originated from a Bartha-vaccine-like strain. *Virology* 2016;491:56–63.
- 23 Papageorgiou KV, Suárez NM, Wilkie GS, Filioyannis G, Papaioannou N, Nauwynck HJ, Davison AJ, Kritas SK: Genome sequences of two pseudorabies virus strains isolated in Greece. *Genome Announc* 2016;4:e01624–15.
- 24 Darriba D, Taboada GL, Doallo R, Posada D: jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 2012;9:772.
- 25 Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 2013;30:2725–2729.
- 26 Huson DH, Bryant D: Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 2006;23:254–267.
- 27 Librado P, Rozas J: DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 2009;25:1451–1452.
- 28 Bandelt HJ, Forster P, Röhl A: Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* 1999;16:37–48.
- 29 Drummond AJ, Rambaut A: BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007;7:214.
- 30 Lemey P, Rambaut A, Drummond AJ, Suchard MA: Bayesian phylogeography finds its roots. *PLoS Comput Biol* 2009;5:e1000520.
- 31 Bielejec F, Rambaut A, Suchard MA, Lemey P: SPREAD: spatial phylogenetic reconstruction of evolutionary dynamics. *Bioinformatics* 2011;27:2910–2912.
- 32 Fonseca A Jr, Cottorello, AC, D'Ambros R, Leite RC, Heinemann MB, Reis JKPR: PCR em tempo real para detecção do vírus da doença de Aujeszky. *Arq Bra Med Vet Zoo* 2013;65:801–808.
- 33 Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids Res* 2001;29:e45.
- 34 Müller T, Bätz HJ, Schlüter H, Conraths FJ, Mettenleiter TC: Eradication of Aujeszky's disease in Germany. *J Vet Med B Infect Dis Vet Public Health* 2003;50:207–213.
- 35 Pannwitz G, Freuling C, Denzin N, Schaarschmidt U, Nieper H, Hlinak A, Burkhardt S, Klopries M, Dedek J, Hoffmann L, Kramer M, Selhorst T, Conraths FJ, Mettenleiter T, Müller T: A long-term serological survey on Aujeszky's disease virus infections in wild boar in East Germany. *Epidemiol Infect* 2012;140:348–358.
- 36 Müller T, Hahn EC, Tottewitz F, Kramer M, Klupp BG, Mettenleiter TC, Freuling C: Pseudorabies virus in wild swine: a global perspective. *Arch Virol* 2011;156:1691–1705.
- 37 Larson G, Dobney K, Albarella U, Fang M, Matisoo-Smith E, Robins J, Lowden S, Finlayson H, Brand T, Willerslev E, Rowley-Conwy P, Andersson L, Cooper A: Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. *Science* 2005;307:1618–1621.
- 38 Ramírez O, Ojeda A, Tomás A, Gallardo D, Huang LS, Folch JM, Clop A, Sánchez A, Badoui B, Hanotte O, Galman-Omitogun O, Makuza SM, Soto H, Cadillo J, Kelly L, Cho IC, Yeghoyan S, Pérez-Enciso M, Amills M: Integrating Y-chromosome, mitochondrial, and autosomal data to analyze the origin of pig breeds. *Mol Biol Evol* 2009;26:2061–2072.
- 39 Sakaoka H, Kurita K, Iida Y, Takada S, Umene K, Kim YT, Ren CS, Nahmias AJ: Quantitative analysis of genomic polymorphism of herpes simplex virus type 1 strains from six countries: studies of molecular evolution and molecular epidemiology of the virus. *J Gen Virol* 1994;75:513–527.
- 40 Verpoest S, Cay AB, Van Campe W, Mostin L, Welby S, Favoreel H, De Regge N: Age- and strain-dependent differences in the outcome of experimental infections of domestic pigs with wild boar pseudorabies virus isolates. *J Gen Virol* 2016;97:487–495.
- 41 Verpoest S, Cay AB, Favoreel H, De Regge N: Pseudorabies virus isolates from domestic pigs and wild boars show no apparent in vitro differences in replication kinetics and sensitivity to interferon-induced antiviral status. *J Gen Virol* 2016;97:473–479.
- 42 Timoneda O, Núñez-Hernández F, Balcells I, Muñoz M, Castelló A, Vera G, Pérez LJ, Egea R, Mir G, Córdoba S, Rosell R, Segalés J, Tomás A, Sánchez A, Núñez JI: The role of viral and host microRNAs in the Aujeszky's disease virus during the infection process. *PLoS One* 2014;9:e86965.
- 43 Flori L, Gao Y, Laloë D, Lemonnier G, Leplat JJ, Teillaud A, Cossalter AM, Laffitte J, Pinton P, de Vaureix C, Bouffaud M, Mercat MJ, Lefèvre F, Oswald IP, Bidanel JP, Rogel-Gailard C: Immunity traits in pigs: substantial genetic variation and limited covariation. *PLoS One* 2011;6:e22717.
- 44 Fang M, Larson G, Ribeiro HS, Li N, Andersson L: Contrasting mode of evolution at a coat color locus in wild and domestic pigs. *PLoS Genet* 2009;5:e1000341.
- 45 Fonseca AA Jr, Heinemann MB, Leite RC, Reis JK: A comparative analysis of envelope and tegument proteins of Suid herpesvirus 1, bovine herpesvirus 1 and bovine herpesvirus 5. *Arch Virol* 2010;155:1687–1692.