

High prevalence of amantadine resistance among circulating European porcine influenza A viruses

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Genetic analysis of the M2 sequence of European porcine influenza A viruses reveals a high prevalence of amantadine resistance due to the substitution of serine 31 by asparagine in all three circulating subtypes, H1N1, H3N2 and H1N2. The M segment of all resistant strains belongs to a single genetic lineage. Whereas the first amantadine-resistant porcine strain was isolated in 1989, isolation of the last amantadine-susceptible strain dates to 1987, suggesting a displacement of amantadine-susceptible viruses by resistant strains soon after emergence of the mutation. Analysis of natural selection by codon-based tests indicates negative selection of codons 30, 31 and 34 which confer amantadine resistance. The codons 2, 11–28 and 54 of porcine and human strains exhibit differences in the patterns of substitution rates, suggesting different selection modes. Transfer of amantadine resistance by exchange of the M segment and viability of recombinant A/WSN/33 viruses with avian-like M segments raises concerns about the emergence of natural human reassortants.

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INTRODUCTION

Influenza A viruses (FLUAVs) are characterized by certain features which make them a unique aetiological agent of a highly dynamic disease. The annual fluctuation of human influenza prevalence in the temperate zone with a peak in winter, the high morbidity and mortality of certain human age cohorts (elderly people above age 65, children) and the threat of a pandemic justify influenza surveillance in the populations of humans and some domestic animals. Additionally, the monitoring of susceptibility to antivirals should receive attention for preparedness for an influenza pandemic. There is serological and phylogenetic evidence that early European virus strains isolated from pigs since 1930 belong to either genetic lineage, human or 'classical swine' H1N1 FLUAV (Nardelli *et al.*, 1978; Brown *et al.*, 1997). In 1979, novel porcine influenza A viruses (swFLUAV) of the H1N1 subtype emerged in Europe (Pensaert *et al.*, 1981). These viruses have eight genome segments originating from avian strains and are designated 'avian-like' accordingly. After the initial bird-to-swine transmission, the European avian-like swFLUAVs underwent subsequent reassortments with human influenza

viruses to yield 'human-like' swFLUAV H3N2 (emergence 1984) and H1N2 subtypes (emergence 1994). Those subtypes have human haemagglutinin (HA) and neuraminidase (NA) segments, whereas the internal segments are avian-like (Brown, 2000). H3N2 and H1N2 reassortants spread region-wide in European swine populations and replaced the classical swine viruses. European swFLUAVs constitute distinct genetic lineages (Brown *et al.*, 1998) and have the propensity to infect humans (Rimmelzwaan *et al.*, 2001; Gregory *et al.*, 2001, 2003; Claas *et al.*, 1994). Recently, the emergence of amantadine-resistant European swFLUAVs and an alarming increase of amantadine-resistant human isolates was reported (Bright *et al.*, 2005; Deyde *et al.*, 2007; Marozin *et al.*, 2002; Schmidtke *et al.*, 2006, 2008). Amantadine, an adamantane derivative, is an antiviral compound effective against FLUAV. Despite certain side-effects and a rapid induction of resistant strains, amantadine is licensed for the prophylaxis and therapy of influenza in various countries (for review, see Hayden, 2006). It inhibits the function of the FLUAV M2 proton channel and single amino acid substitutions at positions 26, 27, 30, 31 and 34 of the M2 protein confer resistance against it (Hay *et al.*, 1985; Abed *et al.*, 2005). Recently, structure determination of tetrameric M2

Supplementary material is available with the online version of this paper.

transmembrane helices in presence and absence of amantadine and rimantadine, respectively, provided evidence for refined models describing both proton gating and viral inhibition by adamantanes (Schnell & Chou, 2008; Stouffer *et al.*, 2008). In this study, a molecular epidemiological investigation of European swFLUAVs, analysis of their amantadine resistance phenotype and a genetic analysis of the M segment was performed. In addition, recombinant viruses which correspond to reassortant human viruses with avian-like M segments of European swFLUAVs were rescued and analysed for their amantadine susceptibility.

METHODS

Viruses. The German swFLUAV isolates were collected between 1981 and 2006 from diseased pigs (a compilation of virus strains is available as Supplementary Table S1, available in JGV Online). Virus was isolated in embryonated hens eggs from nasal swabs obtained from pigs with clinical symptoms. Viruses were passaged on Madin-Darby bovine kidney (MDBK) cells or on Madin-Darby canine kidney (MDCK) cells as described previously (Schmidtke *et al.*, 2006; Zell *et al.*, 2008). Additional virus isolates were obtained from collections of the Bundesinstitut für Risikoforschung (Berlin, Germany), the Niedersächsisches Landesgesundheitsamt (Hannover, Germany) and the Department of Virology, Parasitology, Immunology of Ghent University (Ghent, Belgium).

Virus RNA preparation and sequencing. For sequencing of the viral genome, total RNA was prepared from virus-infected MDCK cells using the RNeasy Mini and QIAshredder kits (Qiagen). Reverse transcription was conducted with a primer specific to the 3'-end of genomic RNA, 20 U reverse transcriptase (Fermentas) and 5 µg RNA in a final reaction volume of 20 µl. Specific oligonucleotide primers (Schmidtke *et al.*, 2006) and cDNA were used for the amplification of DNA employing *Pfu* polymerase (Promega). Amplification products were subjected to agarose gel electrophoresis and extracted employing a QIAquick Gel Extraction kit (Qiagen). Purified amplification products were sequenced by cycle sequencing using the CEQ DTCS Quick Start kit (Beckman Coulter) and analysed on a CEQ8000 sequencer (Beckman Coulter). The nucleotide sequences were submitted to GenBank (accession nos EU478795–EU478849).

Phylogenetic and molecular genetic analyses. Nucleotide sequences were aligned manually with the help of MEGA version 4 (Tamura *et al.*, 2007). For tree inference, Bayesian Metropolis-coupled Markov chain (MCMC) analysis was conducted with MrBayes 3.1 (Ronquist & Huelsenbeck, 2003). Four chains were calculated until convergence was reached (substitution model: GTR + G + I, four gamma categories, chain length: 1 900 000 generations). The optimal substitution model was selected in a hierarchical likelihood ratio test on the basis of the Akaike information criterion (AIC) using MODELTEST (Posada & Crandall, 1998). The tree was visualized with TreeView 1.6.6 (Page, 1996). Divergence times within the European swFLUAVs were inferred using Bayesian MCMC analysis and a relaxed clock model as implemented in BEAST 1.4.7 (Drummond & Rambaut, 2007). The dataset was analysed using the GTR + G + I substitution model, four gamma categories and a chain length of 50 000 000 generations. Two partitions into codon positions (SRD06 model, default unlink parameters) and a relaxed clock (uncorrelated lognormal) were assumed. Log parameters were sampled every 1000 generations. A maximum credibility tree was constructed with FigTree 1.1.2 (Drummond & Rambaut, 2007). Posterior probabilities of nodes, node ages (node heights) and branch

lengths were displayed. Natural selection acting on each amino acid site was inferred using codon-based maximum-likelihood methods (the single likelihood ancestor counting method, SLAC; the fixed effects likelihood method, FEL; and the internal fixed effects likelihood method, IFEL) provided by the Datamonkey web server (<http://www.datamonkey.org>; Kosakovsky Pond & Frost, 2005). Positive selection (non-synonymous substitution rate greater synonymous substitution rate, $dN > dS$) and negative selection ($dN < dS$) was estimated as $dN - dS$ differences. In order to compare the results of different methods, normalized $dN - dS$ values [$(dN - dS) / (\text{codon tree length})$] are presented. Co-evolution of amino acid sites was analysed with the Spidermonkey/BGM tool (Poon *et al.*, 2007) of the Datamonkey web server. This software tool performs a Bayesian graphical model (BGM) analysis on aligned nucleotide sequences. Genotype determination was conducted with the FluGenome web server (<http://www.FluGenome.org>; Lu *et al.*, 2007).

Antiviral testing. The plaque reduction assay was used for antiviral testing. It was conducted as previously described (Schmidtke *et al.*, 2006). Briefly, MDCK cells were infected and overlaid with 0.4 % agar supplemented with 0.001–10 µg amantadine ml⁻¹. At day 3–4, the plaque number was determined and the IC₅₀ was calculated. Rescued viruses were examined for amantadine susceptibility by virus titration in the presence of various amantadine concentrations according to Masuda *et al.* (2000). Amantadine resistance was defined as an at least 50-fold increase of IC₅₀.

Reverse genetics. For the generation of recombinant viruses, the M segments of two H1N1 viruses, A/sw/Potsdam/15/81 (amantadine-susceptible) and A/sw/Belzig/2/01 (amantadine-resistant), were amplified. PCR fragments were purified and cloned into pHW2000 (Hoffmann *et al.*, 2000). The pHW plasmid system allows the rescue of recombinant A/WSN/33 (H1N1). Mixed cultures of 293T/MDCK cells were transfected with mixtures of eight plasmids (total 2 µg DNA) using FuGENE (Roche). Plasmids pHW18-1 to -8 served as positive control. Viable recombinant virus (either reassortant virus or control virus A/WSN/33) was rescued and passaged ten times in MDCK cells to ascertain viability. Reassortant virus, A/WSN/33 and both parental (non-recombinant) viruses were assayed for amantadine resistance phenotype.

RESULTS

Sequence analysis and genotyping of European swFLUAVs

A recent pilot study revealed amantadine resistance in nine out of 12 German swFLUAV strains (Schmidtke *et al.*, 2006). Those viruses belonged either to the avian-like H1N1 or human-like H3N2 and H1N2 subtypes. In order to undertake a more comprehensive analysis, we studied additional swFLUAV strains of all three subtypes that presently circulate in Europe. The isolates were collected in Germany between 1981 and 2006. In addition, one English and three Belgian isolates were included. Because five substitutions of the M2 open reading frame strongly correlate with the amantadine-resistant phenotype (Abed *et al.*, 2005), genotyping, i.e. sequencing of the M segment, was previously used for the analysis of amantadine resistance of human and avian influenza virus isolates (Bright *et al.*, 2005; Deyde *et al.*, 2007; Hurt *et al.*, 2007). Fifty-five M sequences determined in this study as well as additional 49 (almost) complete and eight partial M

sequences retrieved from GenBank were compiled to yield an alignment of M sequences of European swFLUAVs ($n=112$, Supplementary Table S1). This alignment included sequences of eight swFLUAV isolates from Hong Kong as well as one German turkey isolate and two zoonotic human isolates from Hong Kong and Switzerland (Gregory *et al.*, 2001, 2003), since those viruses were found to belong to the genetic lineage of prevalent European swFLUAVs. Sequence comparison revealed that all swFLUAV isolates since 1989 have the characteristic S31N substitution of the M2 protein conferring amantadine resistance (Supplementary Table S1). In addition, double mutants with the S31N and either of the L26F, V27A or V27T substitutions were also observed in some resistant strains. The second substitution also confers amantadine resistance (Hay *et al.*, 1985; Abed *et al.*, 2005). Interestingly, 10 strains have the novel L26I-V27A-S31N triple substitution, another strain has L26I-V27T-S31N substitutions. Yet unknown is the significance of L26I and V27I exchanges. Moreover, a coincident R77Q substitution was observed in all resistant swFLUAV strains. A GenBank

survey revealed that these S31N/R77Q exchanges were not found in any other M sequence of human or animal virus isolates (data not shown).

Antiviral testing

In previous studies amantadine resistance was deduced from genetic data and verified for some (arbitrarily) selected strains by further antiviral testing (e.g. Bright *et al.*, 2005; Deyde *et al.*, 2007; Hurt *et al.*, 2007). In analogy, we confirmed the amantadine resistance phenotype of a representative fraction of our collection (29/70 randomly selected isolates=41%) using the plaque reduction assay as described (Schmidtke *et al.*, 2006). As expected, amantadine resistance correlates with the S31N substitution (Supplementary Table S1): all susceptible strains have a serine codon at position 31, whereas the resistant strains have the S31N substitution. Antiviral analysis confirmed the observation that strains isolated after 1989 are amantadine-resistant, whereas the elder strains are amantadine-susceptible (Table 1).

Table 1. Amantadine susceptibility of porcine influenza A viruses determined in plaque reduction assays in MDCK cells

| swFLUAV subtype | Amantadine susceptibility | Strain | IC ₅₀ ($\mu\text{g ml}^{-1}$) | | |
|------------------------------------|---------------------------|-----------------------------------|--|-----------------------------|------|
| H1N1 | Susceptible | A/sw/Potsdam/1/81 (H1N1) | 0.01 | | |
| | | A/sw/Potsdam/15/81 (H1N1) | 0.05 | | |
| | Resistant | A/sw/Schwerin/103/89 (H1N1) | >25 | | |
| | | A/sw/Bakum/5/95 (H1N1) | 5.00 | | |
| | | A/sw/Belzig/2/01 (H1N1) | >25 | | |
| | | A/sw/Wedel/IDT2965/04 (H1N1) | >10 | | |
| | | A/sw/Kroegel/IDT4192/05 (H1N1) | >10 | | |
| | | A/sw/Laer/IDT3893/05 (H1N1) | >10 | | |
| | | A/sw/Voglarn/IDT4096/05 (H1N1) | >10 | | |
| | | A/sw/Wohlerst/IDT4093/05 (H1N1) | >10 | | |
| | | A/sw/Bakum/1832/00 (H1N2) | >25 | | |
| | | A/sw/Bakum/1833/00 (H1N2) | >25 | | |
| | | A/sw/Gescher/IDT2702/03 (H1N2) | >10 | | |
| A/sw/Löningen/IDT2530/03 (H1N2) | >10 | | | | |
| H1N2 | Resistant | A/sw/Gudensberg/IDT2930/04 (H1N2) | >10 | | |
| | | A/sw/Nortrup/IDT3685/04 (H1N2) | >10 | | |
| | | A/sw/Stolzenau/IDT3296/04 (H1N2) | >10 | | |
| | | A/sw/Dötlingen/IDT3780/05 (H1N2) | >10 | | |
| | | H3N2 | Susceptible | A/sw/Potsdam/35/82 (H3N2) | 0.05 |
| | | | | A/sw/Karrenzien/2/87 (H3N2) | 0.06 |
| | | Resistant | A/sw/Leipzig/145/92 (H3N2) | >25 | |
| | | | A/sw/Jena/5/96 (H3N2) | >25 | |
| | | | A/sw/Lohne/1/97 (H3N2) | 16.4 | |
| | | | A/sw/Belzig/54/01 (H3N2) | >10 | |
| A/sw/Bakum/IDT1769/03 (H3N2) | >10 | | | | |
| A/sw/Bakum/8602/99 (H3N2) | >10 | | | | |
| A/sw/Harkenblek/IDT4097/05 (H3N2) | >10 | | | | |
| A/sw/Nordkirchen/IDT1993/03 (H3N2) | >10 | | | | |
| A/sw/Damme/IDT2890/04 (H3N2) | >10 | | | | |

Phylogenetic analysis

Phylogenetic analysis was based on 113 (almost) complete M sequences including nine sequences representing eight M genotypes for comparison. Representatives of the genotypes A–G were selected from a collection of the FluGenome web server (Lu *et al.*, 2007). These sequences served as outgroups for the phylogenetic analysis and represent genetic lineages found in (i) pigs (genotype A: classical swine), (ii) humans (genotype B), (iii) gulls (genotype C), (iv) horses (genotype D), (v) American birds (genotype E), (vi) Eurasian/Australian birds (genotype F) and (vii) a few isolates from Australian stints represent genotype G. In addition, one sequence (A/shorebird/Delaware/168/2006, H16N3) of a yet unclassified genotype was also included. Beside these sequences, the dataset comprised 105 available sequences (55 sequences determined in this study and 50 sequences of avian-like isolates retrieved from GenBank). A phylogenetic tree was inferred using Bayesian MCMC analysis. The resulting tree (Fig. 1) indicates that the M sequences of the European swFLUAVs constitute a monophyletic branch within the F genotype. This clade also includes sequences of one avian strain (A/turkey/Germany/3/91, H1N1), eight porcine H3N2 strains from Hong Kong (isolated 1999–2002) and one human isolate (A/Hong Kong/1774/99, H3N2). Additional phylogenetic analyses verified that all published sequences of these viruses clustered with European swFLUAVs (data not shown). Another avian-like isolate, A/sw/Hong Kong/168/1993 (H1N1), is clearly distinct from the European lineage of swFLUAVs, but clusters within the F genotype. Surprisingly, all amantadine-resistant swFLUAV strains (as deduced from the S31N substitution) cluster in a single clade with A/sw/Schwerin/103/89 (H1N1) being closest to the root. The respective node is supported with high posterior probability (1.0). Monophyly of the European swFLUAV lineage was verified using another alignment comprising sequences of the European swFLUAVs and additional 20 arbitrarily selected avian sequences of the F genotype (data not shown). Beside the S31N mutation, additional substitutions (A26F, L26I, V27A, V27I, A27T) could be traced. None of them emerged independently of the S31N substitution.

Divergence times were estimated for the clade of European swFLUAVs. The years of isolation of the swFLUAV strains served as calibration points. The data presented in Fig. 2 were derived from a relaxed clock analysis and reveal that the swFLUAV lineage appeared in 1979 [node age: 26.83 years before 2006, 95% highest posterior density (HPD) interval: 26.14–33.39 years before 2006) and amantadine-resistance (S31N substitution) may have emerged in 1988 (node age: 17.8 years before 2006, range of the 95% HPD interval: 17.09–22.55 years before 2006). 1979 as the year of emergence of European swFLUAVs was also inferred by Ludwig *et al.* (1995).

In order to find evidence for co-evolution between S31N (AGT to AAT) and R77Q (CGA to CAA), a BGM analysis

was conducted using an alignment of 71 unique M2 sequences (294 nt) of the European swFLUAV lineage. This analysis supported both substitutions being conditionally dependent at a significant level (posterior probability: 0.89).

Natural selection of the M2 open reading frame

Codon-based tests were performed to identify natural selection at single amino acid sites. Positive (diversifying) selection, i.e. excess of non-synonymous substitutions, and negative (purifying) selection, i.e. excess of synonymous substitutions, were investigated using different maximum-likelihood methods (SLAC, FEL and IFEL). Several codons were suggested to be restrained by negative selection: (i) codons 30, 31 and 34 which confer amantadine resistance and (ii) codons 32, 50, 51, 58, 67 and 71 (Supplementary Table S2, available in JGV Online). Positive selection was suggested for codon 19 in the IFEL method. The corresponding *P*-values were <0.05, indicating that less than 5% of neutrally evolving sites may be incorrectly classified as selected. All three methods showed a similar pattern of *dN*–*dS* differences. However, the SLAC and IFEL methods were found to be more conservative, resulting in fewer suggestions for natural selection (Fig. 3, left panel). In addition, 46 of 97 codons (47.4%) were invariant. The mean substitution rate of this dataset was 0.54 substitutions/site and the mean *dN/dS* was 0.60. For comparison, a dataset comprising 80 unique M2 sequences of human H1N1 and H3N2 influenza virus isolates was also included in this analysis (Supplementary Table S2, Fig. 3, right panel). The mean substitution rate of this dataset was slightly greater (0.67 substitutions/site), but the mean *dN/dS* value was smaller (0.44) indicating some more synonymous substitutions. For this dataset, positive selection of codons 28, 36, 54, 89 and 93 and negative selection of codons 2, 25, 29, 30, 32, 34, 35, 45, 51, 53, 58, 59, 65, 69, 71, 75 and 96 was suggested; 33% of the codons were invariant. Negative selection of codons 30, 32, 34, 51, 58 and 71 was suggested in both datasets, but the overall patterns of the *dN*–*dS* values along the open reading frames differ at codon 2, 11–28 and 54 (Fig. 3), indicating diverse selective forces which act on the M2 proteins of porcine and human influenza A viruses.

Transfer of amantadine resistance by exchange of the M segment

As indicated in Fig. 1, the M segment of human and porcine influenza viruses belong to different genotypes. Therefore, the question arose whether a reassortant A/WSN/33 virus with a M segment of genotype F rather than genotype B is viable. As a proof of principle, the compatibility of the avian-like M segment, the transfer of the amantadine resistance phenotype and the viability of such a potential reassortant virus was demonstrated. For the generation of recombinant viruses, the M segments of an amantadine-susceptible strain (A/sw/Potsdam/15/81,

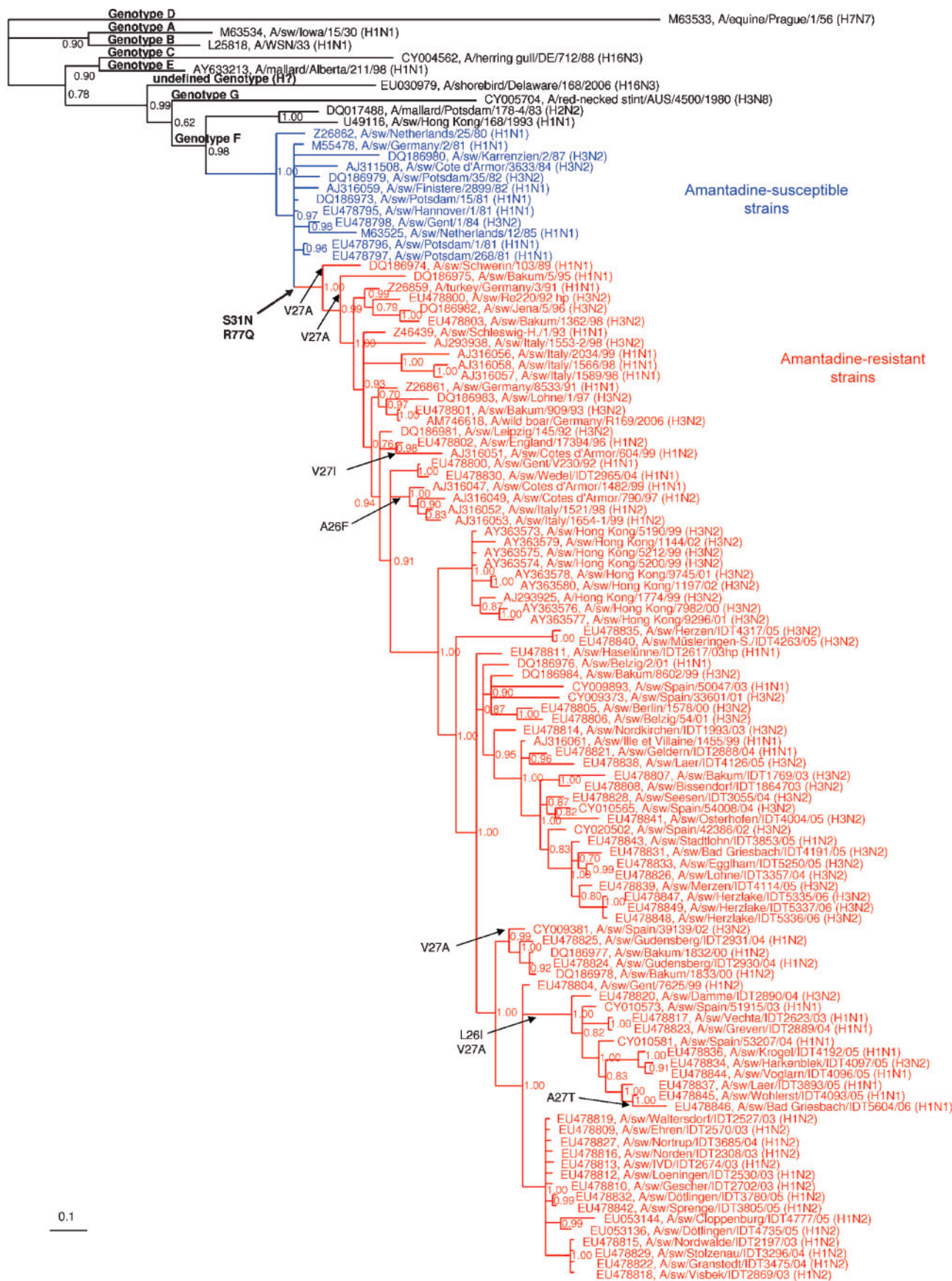


Fig. 1. Phylogenetic analysis of 113 FLUAV M gene segments (compare with Supplementary Table S1, available in JGV Online). Sequences (982 nt) were aligned and used to infer a tree with Bayesian statistics. Reference sequences of other genotypes were included (A, classical swine isolate; B, human isolate; C, isolate from American gull; D, equine isolate; E, American avian isolate; F, Eurasian/Australian avian isolate; G, isolate from Australian shorebird; H, genotype not defined by the FluGenome web server). Names of strains and the accession numbers of the respective GenBank entries are presented. The sequence of *A/equine/Prague/1/56* (H7N7) serves as an outgroup. Blue and red colours indicate amantadine-susceptible and amantadine-resistant swFLUAV strains, respectively. The thick arrow indicates the branch with the S31N/R77Q mutations and thin arrows point to clades with additional mutations of amantadine-binding amino acids. All M sequences of European swFLUAVs belong to genotype F. Bar, 0.1 substitutions per site. Numbers at nodes indicate posterior probabilities obtained after 1 900 000 generations.

H1N1) and an amantadine-resistant strain (*A/sw/Belzig/2/01*, H1N1) were cloned into pHW2000 (Hoffmann *et al.*, 2000) and two recombinant A/WSN/33 viruses (H1N1) were rescued using the eight plasmid system previously described (Hoffmann *et al.*, 2000). These viruses were designated A/WSN/33xA/sw/Potsdam/15/81-M (amantadine-susceptible) and A/WSN/33xA/sw/Belzig/2/01-M (amantadine-resistant). Subsequently, the rescued viruses were tested for viability in serial passages and the amantadine susceptibility was assayed by determination of the mean dose–response curves. They demonstrate that, independently of the genomic background, the M segment of *A/sw/Potsdam/15/81* confers amantadine sensitivity ($IC_{50} < 0.25 \mu\text{g ml}^{-1}$), whereas the M segment of *A/sw/Belzig/2/01* leads to amantadine resistance ($IC_{50} > 10 \mu\text{g ml}^{-1}$) (Fig. 4).

DISCUSSION

In this study, we investigated the amantadine susceptibility of European swFLUAVs. Their M segments comprise a

distinct clade within genotype F. All resistant swFLUAVs have the S31N substitution, which prevents binding of amantadine to the M2 protein. Several isolates have an additional V27A substitution, which affords resistance by another mechanism: though amantadine-binding is retained, M2 protein still functions as a proton channel (Astrahan *et al.*, 2004). The significance of the L26I substitution, which is also found in amantadine-resistant H5N1 isolates from Vietnam, Thailand, Cambodia and Malaysia of 2004–2005 (Li *et al.*, 2004; Hurt *et al.*, 2007) is still unclear. Two further observations are noteworthy: (i) the emergence of amantadine resistance of swFLUAVs was a single event as indicated by the monophyly of the resistance-conferring M segment; (ii) subsequently, amantadine-susceptible H1N1 viruses were displaced by resistant strains. As concluded from the phylogenetic tree, the resistance-conferring M segment was then transferred to H3N2 viruses by reassortment. The earliest resistant H3N2 strain was isolated in 1992. Then, in 1994, the first H1N2 strains emerged in the UK; those strains resulted from reassortment (Brown *et al.*, 1998) and are amantadine-resistant. Due to the few sequences of that time available in

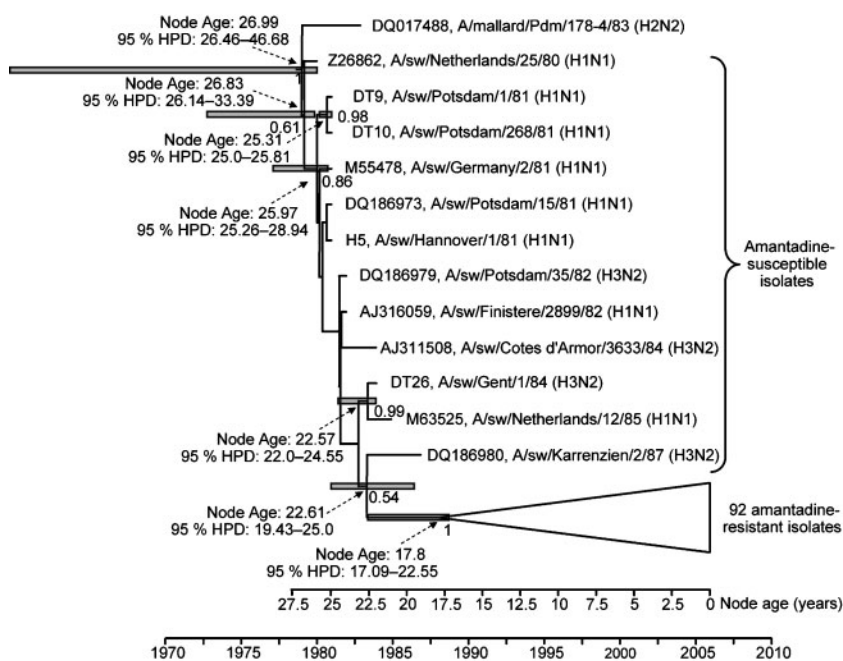


Fig. 2. Divergence times estimate of the European swFLUAV branch. *A/mallard/Pdm/178-4/83* (H2N2) serves as an outgroup. Numbers at nodes indicate the posterior probability of the respective nodes. Node bars illustrate the 95% highest posterior density (HPD) intervals for each divergence time. Node ages and values of the 95% HPD intervals for nodes with significant support (posterior probability > 0.5) are presented. The branch of 92 amantadine-resistant isolates was collapsed for clarity. Branch lengths corresponds to years. Node age 0 corresponds to the year of isolation of the youngest strain (isolated in 2006).

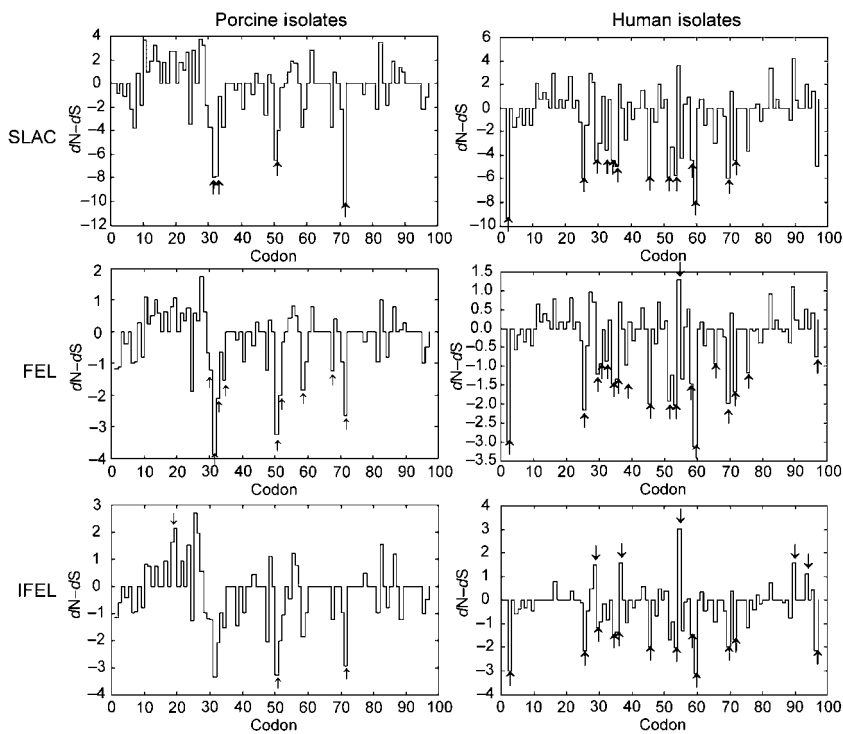


Fig. 3. Natural selection acting on porcine (left panel) and human influenza virus strains (right panel). Results of three different maximum-likelihood methods (SLAC, FEL, IFEL) are presented. Arrows indicate codons with significant statistical support (P -value < 0.05). Note that $dN-dS$ differences of invariant codons cannot be estimated and are set to 0.

GenBank, the exact date of the S31N/R77Q substitutions and the space of time needed for the successive replacement of the amantadine-susceptible strains remain obscure, but it is very likely that this time span might have been short. The molecular clock analysis indicates that the S31N substitution might have occurred in 1988 (95 %

HPD: 1983–1989). In fact, no sequenced European swFLUAV isolated since 1989 is amantadine-susceptible, whereas none of the viruses of 1979–1987 is resistant (Fig. 1, Supplementary Table S1). Since 92 amantadine-resistant strains were collected by different laboratories throughout Europe over a period of 17 years, biased sampling that favoured isolation of resistant strains is highly unlikely. The sequences of the present study are predominantly from German isolates, but Belgian, British, Dutch, French, Italian, Spanish, Swiss and Hong Kong strains were also included in order to compile the most comprehensive collection of M sequences currently available.

Spontaneous emergence of amantadine-resistant porcine influenza viruses in the absence of the drug is a rare event. Worldwide, only three independent incidents have been documented. Beside the emergence in Europe, only two clades were found in an analysis of available sequence data (approx. 180 sequences) of classical swine isolates (H1N1) and reassortant H1N2, H3N2 and H3N1 strains derived thereof (data not shown). As there are no indications that pigs ever have been treated with amantadine in Europe, the emergence of amantadine resistance has very likely a natural cause. The reason for the astounding spread of resistant porcine virus strains remains elusive, but may not be unusual. In recent years, the prevalence of adamantane-resistant human influenza viruses has been increasing rapidly (compare Bright *et al.*, 2005; Deyde *et al.*, 2007). Previous investigations indicate that two mechanisms may account for the amantadine resistance of human FLUAVs: spontaneous substitutions occurring with low incidence (Ziegler *et al.*, 1999), and the selection of resistant variants

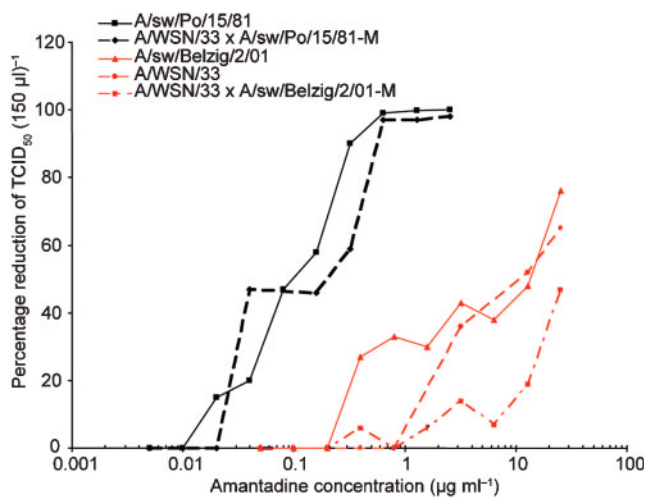


Fig. 4. Mean dose–response curves determined with 0.005–25 μg amantadine ml^{-1} by TCID_{50} assay in MDCK cell monolayers infected with wild-type (A/sw/Potsdam/15/81, A/sw/Belzig/2/01, A/WSN/33) and rescued influenza viruses (A/WSN/33xA/sw/Potsdam/15/81-M, A/WSN/33xA/sw/Belzig/2/01-M).

upon antiviral therapy (Shiraishi *et al.*, 2003). Interestingly, excessive use of adamantane inhibitors may not explain upsurge of resistant human strains in many countries. Simonsen *et al.* (2007), observed that a 4+4 segment reassortment event favoured resistant strains due to fitness-enhancing mutations at other genomic sites. For swFLUAVs, the sequence data do not allow the demonstration of such a hitchhiking effect (data not shown). Also, selection of resistant strains upon antiviral therapy appears to be an unlikely mechanism, but a bottleneck effect might be a reasonable explanation (Domingo & Holland, 1997).

Our codon-based tests for natural selection revealed that codons 30, 31 and 34, which are known to confer amantadine resistance, are likely to be negatively selected. For codon 31, it appears that only four states are allowed: either AG^T/_C (encoding serine; amantadine-susceptible) or AA^T/_C (encoding asparagine; amantadine-resistant). The remaining four serine codons (UCA, UCG, UCC, UCT), though coding for the wild-type amino acid, were not observed – neither in porcine nor in human influenza viruses. One may argue that the S31N substitution is the only viable mutation at this site which maintains both structural constraints (i.e. formation of a proper transmembrane helix which is able to tetramerize) and functional constraints of the M2 protein (i.e. proton channel activity even in the presence of amantadine). Substitutions of alanine 30 and glycine 34 were not observed in the swFLUAVs (Supplementary Table S1).

A concerning issue is the possibility that the swFLUAV M segment might be introduced into human influenza viruses by reassortment. In order to substantiate the biological significance of this hypothetical threat, we demonstrate the viability of A/WSN/33 reassortants with M segments of European swFLUAVs. Accordingly, the amantadine susceptibility of the reassortant was altered (Fig. 4), indicating amantadine resistance *in vivo*. Whereas the M segment of the European swFLUAVs belongs to genotype F, all human subtypes have a M segment of the B genotype (Fig. 1). The viability of our rescued H1N1 reassortant along with its amantadine resistance suggests a possible scenario in which the M segments of human viruses could be replaced by an avian-like M segment in the field. Beside circulating amantadine resistant human H3N2 and H1N1 viruses, human–swine reassortants with an avian-like M segment could be an additional, however yet unforeseeable menace. A similar concern was also expressed by Claas *et al.* (1994) and Gregory *et al.* (2001). Therefore, both intense surveillance and elucidation of the acting molecular selection mechanisms are indispensable.

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