Expanded co-circulation of stable subtypes, emerging lineages and new sporadic reassortants of porcine influenza viruses in swine populations in Northwest Germany

Running title: Emerging porcine influenza viruses in Germany

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Abstract

The emergence of the human 2009 pandemic H1N1 (H1N1pdm) from swine populations refocused public and scientific attention towards swine as an important source of influenza A viruses bearing zoonotic potential. Widespread and year-round circulation of at least four stable lineages of porcine influenza viruses in Germany in a region with a high density swine population between 2009 and 2012 is documented here. European avian-derived H1N1 (H1N1av) viruses dominated the epidemiology, followed by human-derived subtypes H1N2 and H3N2. H1N1pdm viruses and, in particular, recently emerged reassortants between H1N1pdm and porcine HxN2 viruses (H1pdmN2) were detected in about 8% of cases. Further reassortants between these main lineages were diagnosed sporadically. On-going diversification both at the phylogenetic and the antigenic level was evident for the H1N1av lineage and for some of its reassortants. The H1avN2 reassortant R1931/11 displayed conspicuously distinct genetic and antigenic features and easily transmitted from pig-to-pig in an experimental infection. Continuing diverging evolution was also observed in the H1pdmN2 lineage. These viruses carry seven genome segments of the H1N1pdm virus including a hemagglutinin gene which encodes an antigenically markedly altered protein. The zoonotic potential of this lineage remains to be determined. The results highlight the relevance of surveillance and control of porcine influenza virus infections. This is important with respect to health status of swine herds. In addition, a more exhaustive tracing of the formation, transmission and spread of new reassortant influenza A viruses with unknown zoonotic potential is urgently required.

Keywords

Swine, influenza virus, surveillance, reassortment, zoonotic potential
Introduction

Influenza A virus (IAV) infections cause economically important diseases in swine populations (1). In piglets and fattening pigs benign respiratory forms of disease often prevail, yet they exert a negative impact on weight gain rates of affected animals. Influenza virus infections in sows may take a highly febrile course and have been considered as a cause of prostaglandin-induced abortions and further fertility disorders. Control of swine influenza is difficult and depends on structural optimization of herd management as well as a strategic use of highly efficacious vaccines (2, 3).

Until recently, in Germany, typical influenza-associated health problems affected swine herds preferably during the cold period of the year, resembling, although unlinked to, seasonal human influenza. Within the last years porcine influenza peaked especially in large industrial sow herds. This went along with increasing rates of return to estrus and abortions affecting sows and particularly gilts after service.

There is a close historic connection of human and porcine influenza viruses. Interestingly, viruses were usually seeded by the human population into swine herds. With the only exception of the Asiatic influenza pandemic of the late 1950s (H2N2) all pandemic human IAVs found their way into swine populations. They adapted to the porcine host and continued to circulate independently from viruses in the human population while evolving into antigenically and phylogenetically discernible porcine lineages (4). Viruses of porcine lineages showed a reduced tendency for antigenic drift compared to their counterparts in the human population (5). In addition to human sources, porcine influenza viruses also originated from avian precursor viruses: A virus lineage of subtype H1N1 of purely avian origin established endemic infection in swine in Europe since the late 1970s. This lineage (H1N1av) dominated the epidemiology in European swine populations until very recently (5). Reassortants of this virus with other human or human-derived lineages gave rise to H1N2 and H3N2 lineages in swine in Europe.

Serosurveys in swine have shown co-circulation of these three enzootic subtypes in different European countries at different prevalences (6). In addition, several reassortants between these lineages have sporadically been detected but no further spread of any of these has been reported (7).

The most recent human pandemic influenza virus, H1N1, which emerged in 2009 (H1N1pdm) and harbors a multi-reassortant genome with several segments of porcine origin, is now also
considered endemic in swine populations in several countries worldwide including many European ones. It already served as a new partner for reassortment with enzootic porcine lineages of subtypes H1N1av, H1N2 and H3N2 (8). In Germany, H1N1pdm was first detected in a swine herd in December 2009. A reassortant virus carrying seven genome segments of the pandemic H1N1 virus and the neuraminidase of H1N1av (H1pdmN1) has been reported in May 2010 (9). Since early 2011 re assortants of subtype H1pdmN2 were found to circulate in five swine herds in Germany over a period of more than one year (10). The hemagglutinin (HA) of these reassortants revealed characteristic unique coding mutations which set them apart from any other HA of pandemic/2009 origin.

Control of porcine influenza by vaccination is based on inactivated adjuvanted vaccines although several studies reported success with recombinant protein, DNA, and live-attenuated vaccines (2). Efficacy of an IAV vaccine depends on a close antigenic match between field and vaccine viruses and is challenged continuously by antigenic shift and drift of field viruses. In addition, interference of maternal-derived antibodies can cause vaccination failure in piglets (11, 12). Antigenic drift of porcine influenza viruses in swine populations appears to be slower compared to influenza viruses in humans (13). This is probably due to the fact that the major part of the swine population is regularly removed at 6-8 months of age due to slaughtering. Replacement with immunologically naïve piglets susceptible also to antigenically “old” influenza virus strains allows for continuing circulation of antigenically stable virus strains. Increasing vaccination rates of sows, observed during recent years in Germany, give rise to a stronger immunological pressure that eventually provokes accelerated antigenic drift of porcine IAV.

The current investigation was planned as a passive monitoring study for porcine influenza viruses in swine in the Northwest of Germany. In a first phase (2009-2010, study A), lung tissues and nasal swabs obtained from swine with severe respiratory disease and/or fertility problems were examined. In a second phase, between March 2011 and March 2012 (study B), nasal swabs from swine holdings with a record of IAV infections despite continuous vaccination of at least a part of the holding’s swine population (usually the sows) were studied. Insight into the dynamic and on-going viral evolution of swine influenza in an area with a pig population of high density was gained by characterizing virus subtypes with respect to their current phylogenetic and antigenic diversification. Experimental infections of swine with representative isolates of new reassortant lineages revealed virulent properties for their natural hosts.
Material and Methods

Study outline and sampling strategy

A. Lung tissues

Data of this study were established by molecular characterization of samples collected between 2009-2010 in Northwest Germany, a region in which 55% of the German swine population (27 million pigs) are housed. Sampling focused on pigs with a history of respiratory disorders in herds with a size of 120 to 650 sows. Specimens of lung tissues obtained during postmortems predominantly of weaners with clinical signs of respiratory symptoms were collected irrespective of further epidemiological stratifications.

B. Nasal swabs

Between March 2011-2012 a targeted sampling approach was followed in swine herds with pronounced respiratory and fertility problems. The study was mainly restricted to the same area as in study A.

Selection criteria for herds were as follows:

− Herd size exceeds ≥ 200 sows; piglets are sold for fattening or fattened on the farm, and
− rate of return to estrus is higher than 15%, and
− herds have been regularly vaccinated against influenza during the preceding 12 months,

and

− problems with sows showing fever and reduced feed intake independent of the period of early lactation.

Selection criteria for animals within such herds comprised:

− Sows: Rectal temperature > 40°C independent of early lactation, reduced feed intake
− Weaners: Clinical signs of acute respiratory disease.

Nasal swabs were to be taken by the consulting veterinarian from up to 15 diseased sows and up to 15 healthy sows in the same bay. In addition, or alternatively, nasal swabs were taken from up to 20 acutely diseased weaners. A commercial swab system was used (Virocult, M&W) which was provided to the veterinarians together with a specific submission form. With this form, consulting veterinarians were also asked to agree to have a telephone interview to further specify the clinical and epidemiological situation of the sampled herd. Samples were submitted without delay at ambient temperature for molecular and virological analysis.
Questionnaire

Telephone interviews with consulting veterinarians who approved their consent on the submission form (comprising exclusively samples for study B) were carried out on basis of a pre-tested and validated questionnaire targeting numerous epidemiological parameters (available on request). Questionnaire data were recorded with Infopath® and descriptive statistics were calculated in Excel®. An English translation of the questionnaire is added as supplemental material.

RNA extraction and detection of viral RNA

RNA was extracted manually as previously described (10) using the Viral RNA kit of Qiagen. Alternatively, samples were extracted semi-automatically by use of the MagAttract Virus Mini kit and a Biosprint96 device (Qiagen). Lung sample homogenates were processed by use of the NucleoSpin 96 Virus Core Kit (Macherey-Nagel) and the freedom evo pipetting robot (Tecan) following the recommendations of the manufacturer. Extracted RNA was then subjected to detection of a fragment of the M gene of the influenza virus RNA genome by real time RT PCR (M1.2 RT-qPCR) employing a modified method described by Fereidouni et al. (14). An “internal” control RNA (IC-2) was used to exclude false-negative results due to PCR inhibition (15). Cq values ≥ 40 were considered negative.

Subtype identification by partial sequence analysis

Samples with Cq values ≤ 33 in the M-gene specific RT-qPCR were selected for subtype characterization of hemagglutinin (HA) and neuraminidase (NA) genes. Amplificates of expected size obtained in conventional RT PCRs using “Pan HA” and “Pan NA” primers as described by Gall et al. (16, 17) were cycle-sequenced and sequences were subjected to a BLASTN2 search of public databases for subtype identification.

Virus isolation

Samples with Cq values ≤ 30 in the M-gene specific RT-qPCR were selected for virus isolation in MDCK cell cultures grown in 6-well plates as described elsewhere (18). Supernatant of cultures showing cytopathic effects until 72 hours of incubation at 35°C were sub-passaged once in MDCK cells in 25 cm² culture flasks. The hemagglutination titer in supernatant was measured following a freeze-thaw cycle and clarification of debris. The HA and NA subtypes of the isolate were confirmed by sequencing as described above. Hemagglutination inhibition (HI) assays were not
Full-length sequencing and phylogenetic analysis of HA, NA and “internal” genes

Selected virus isolates or nasal swabs which were highly positive for influenza virus RNA in M-specific RT-qPCR (Cq values ≤ 25) were used to determine and analyze full length HA and, for a lesser number of samples, NA gene sequences (Table S1a). In general, primers reported by Hoffmann et al. (19) were used for full length amplification of HA, NP, NA, M, and NS genes using the one-step superscript III amplification kit as recommended by the manufacturer (Invitrogen, Darmstadt, Germany). PB1, PB2, and PA gene segments were amplified each in two overlapping fragments using primers described by Li et al. (20). Amplification and sequencing was limited to the HA1 fragment of the HA gene (primer sequences available on request) when RNA from clinical samples had to be used. Sequences were assembled using the GCG software suite and then submitted to the EpiFlu database (accession numbers and isolate identifications listed in supplemental Table S1a and b).

Phylogenetic analysis of HA and NA gene segments was based on alignments of the open reading frames of the HA1 fragment (nucleotides 1-1032, representing amino acids 1-344) and of the full length NA (1401 nucleotides, representing 467 amino acids). Alignments of newly established sequences and selected data base entries were calculated using the “MAFFT” algorithm (21) and further optimized by manual editing using JalView (22). The Akaike criterion calculated by “JModeltest2” (23) was used to choose the most appropriate mutation models. Phylogenetic analyzes consisted of a maximum likelihood approach (“PhyML”, accessed via the ACTG server (24)). The resulting tree topology was used to identify clusters and the most appropriate outgroup sequences for each cluster. The re-ordered alignments were then subjected to an analysis in a Bayesian framework (software suite “MrBayes”) following in principle the guidelines of Smith et al. (25). Mid-point rooted trees were extracted and drawn using “FigTree” software (http://tree.bio.ed.ac.uk/software/figtree/). Further editing of the graphics was carried out with “Inkscape” (http://inkscape.org/).

Experimental infection of swine

Groups of three 13-week old mini pigs each were experimentally infected with porcine influenza virus isolates R2035/11 (H1pdmN2) and R1931/11 (H1avN2). Animals were derived from a breeding herd at FLI. All experiments had received legal approval by an ethics commission (LALLF M-
V/TSD/7221.3-2.5-004(10). Pigs tested seronegative for influenza NP-specific antibodies in a commercial blocking ELISA (IDVet, Montpellier, France). Two pigs of each group were nasally infected by a dose of $10^6$ TCID$_{50}$ in 1 ml of cell culture supernatant. A nebulizer device (Wolfe Tory Medical, Salt Lake City, Utah) was used for instigation. At day 1 post infection (p.i.) a third pig was added as a sentinel of pig-to-pig transmission. Animals were observed clinically daily over ten days and rectal temperatures were recorded once daily. Nasal swab samples were taken at days 2, 4, 7, 10 and 14 p.i. and examined by M-specific RT-qPCR. Blood samples were collected on days 0, 7, 10, 14 and 20 p.i. and examined by NP-specific blocking ELISA and hemagglutination inhibition using homologous antigen (see below). The animals were euthanized on day 24 p.i.

Hemagglutination inhibition assay (HI)

Selected virus isolates were used for antigenic characterization by HI. A panel of previously established post-infection sera from pigs (day 28 p.i.) and ferrets (day 21 p.i.) was used (10, 18). HI procedures followed the method described by Lange et al. (18) including treatment with bacterial neuraminidases. HI results were analyzed by hierarchical agglomerative clustering using the HCE3.5 software package (26, 27) to produce a gridded heat map of normalized titers and a two-dimensional dendrogram representing antigenic relationships. Two-dimensional hierarchical clustering for rows and columns by the complete linkage method was based on an Euclidian distance matrix.

Results

Incidence of porcine influenza virus infections in selected swine herds in Northwest Germany

In the frame of study A, 401 lung tissue specimens obtained mainly from weaners yielded 86 (21.4%) samples positive by M-specific RT-qPCR of which 36 could be further subtyped as shown in Table 1. A total of 2696 nasal swabs obtained from 382 pig herds originated from study B between March 2011 – March 2012 of which 40.3% of holdings and 24.4% of samples tested positive for IAV RNA by M-specific RT-qPCR. Year-round virus circulation independent of the season was observed (Figure 1). Most of the positively tested sample sets from one holding held at least one or two samples with
Cq values ≤ 33 which was a prerequisite for successful molecular subtyping or virus isolation, respectively. However, for roughly a quarter of holdings with positive samples no subtyping results were obtained due to low amounts and/or bad quality of viral RNA. A further 10%, particularly lung tissue samples, could only be partially subtyped (Table 1). Among the fully subtyped samples avian-derived H1N1 viruses dominated clearly (62.7%) followed by subtypes H1N2 (17.6%) and H3N2 (11.1%). Pandemic H1N1 viruses were detected only on two holdings (1.3%) but reassortants between H1N1pdm and porcine HxN2 viruses were present more frequently (H1pdmN2: 7.2%). These reassortants emerged in 2011 and continued to circulate in 2012 (Table 1).

Questionnaire analysis (study B)

In the frame of the targeted study B 236 submissions from sow-keeping farms were received of which a total of 190 questionnaires could be finally included in an evaluation. Sample size is generally small so that false negative results cannot be firmly excluded. Since the majority of sample submissions was from either sows or weaners evaluation was separated as well (sows, n= 123 submissions; weaners, n= 80). Only 15 submissions contained samples from sows and weaners of the same holding. Few herds (n= 19) had been sampled more than once (17 of them twice, one each 3- and 5-times, respectively); these were ignored as repeated sampling and rather treated as a separate sample set in the subsequent statistical description.

A total of 823 samples came from sows of which 48 (5.8%) were influenza virus positive (13/123 herds, 10.6%). Only two of the positive herds originated from older sows, and in four further positive herds the age of the animals (gilts or older sows) was not specified. In seven cases the positive samples were collected from gilts only. Subtyping revealed that most of these herds (n=7) harbored H1N1av virus; H1N1pdm, H1N2 and H3N2 were each detected once while in the remaining three herds no complete subtyping was possible due to low concentrations of viral RNA.

A total of 562 samples (comprising 80 herds) from weaners produced 181 (32.2%) positive samples (43 herds, 53.8%). Again, H1N1av viruses made up the majority (n=17 herds) while H1N1pdm, H1N2 and H3N2 were detected at lower frequencies (4, 5, and 4 herds, respectively). In one case each simultaneous infection with H1N1av/H1N2 or H1N1av/H3N2 were detected at the same time on the holding but in different animals. Incomplete subtyping was recorded for 12 herds. The majority of positive samples originated from weaners aged 7-8 weeks.

Few factors analyzed via the questionnaire data appeared to be correlated (F-test, significance level <0.05) with reduced risks of influenza virus infection (supplemental Table S2). In sows the
most significant factor appeared to be vaccination against PRRSV \((p=0.002)\). Reduced frequencies of influenza virus infections were also seen in weaners which received a circovirus (PCV-2) vaccination \((p=0.001)\) and in holdings which kept their diseased weaners and retardedly growing piglets separated in sick bays \((p=0.007)\).

**Phylogenetic diversity of porcine hemagglutinin (HA1) sequences from Northwest Germany**

The PhyML analysis confirmed presence of three clearly distinct clusters within German porcine \(H1\) sequences representing descendants of the human seasonal \(H1\) (H1N2), the 2009 pandemic \(H1\) (H1pdm) and the avian-derived European porcine H1av (Figure 2a-c, shaded inserts). Separate Bayesian analyses of each of these clusters unravelled distinct lineages within H1pdm and H1av co-circulating in swine in Northwest Germany. A segregation into two separate lineages of the HA1 of H1pdmN2 reassortants within the cluster of H1pdm sequences (Figure 2a) had been noticed earlier (10). This process continued in 2011 and 2012 (e.g., isolates R461/12, R509/12, Figure 2a). HA1 sequences of these reassortant viruses were distinguished from “classical” pandemic \(H1\) by a set of six unique coding mutations (G172E, I183V, S200P, S/T202N, D204S, V338I).

More complex clustering patterns were evident in HA1 sequences of the porcine H1av viruses (Figure 2b). At least four sub-lineages were distinguishable among recent H1av isolates from Germany. Two of these clusters were characterized by unique sets of coding mutations separating them from vaccine strain A/swine/IDT2617/2003 (H1N1) which has generally been used for vaccination on holdings sampled in study B. Isolate R681/12, at the tip of cluster 1, accumulated 10 amino acid exchanges (N52T, S100P, N101D, A106T, K147R, V151I, S179K, I282V, H288R, E319K). Isolate R1856/11, at the tip of the cluster 4, revealed substitutions V7I, T149S, A158I, G172K, L178I, K180T, H288N (7 unique exchanges). Further groups of isolates as well as single isolates with fewer unique mutation patterns were noticed as well. This refers in particular to isolates positioned between clusters 1 and 2 (Figure 2b) which show comparatively long horizontal branches (R369/09, R848/11, R1931/11, R3310/12). Two of these isolates also carry a reassortant NA of subtype N2.

In contrast to the H1pdm and H1av lineages HA1 sequences of the human-derived (hu) H1N2 porcine viruses recently detected in swine in Germany appeared to form a nearly homogenous phylogenetic group with only shallow furcations (Figure 2c). Isolate R 1421/10 was an exception and clustered outside this group. A PhyML analysis which included a wider selection of HA1 sequences of European H1N2 porcine isolates, revealed a peculiar geographic restriction: Contemporary viruses from France/UK, Italy and Germany formed distinct lineages and R1421/10
clustered with older viruses from Central Europe (supplemental Figure 1). Such regional patterns were not observed in PhyML analyses of H1av or H1pdm sequences from European swine (not shown).

Recent porcine H3 HA1 sequences from Germany fell into a separate, slightly fissured cluster among European porcine H3 viruses (Figure 2d). Unique coding mutations separated this lineage from older European porcine H3 isolates (N69K, K315R,) and from each other (blue lineage in Figure 2d – V14G, G16S, D18S, D48N, L127I, V239I).

Phylogenetic diversity of porcine neuraminidase sequences from Northwest Germany

Clustering tendencies were also present in the NA phylogenetic trees. Porcine H1N1av N1 lineages (N1av) were clearly separated from truly avian ones as well as from human seasonal and classical porcine lineages (Figure 3a). The N1 of the human pandemic H1N1 viruses (N1pdm) formed a monophyletic group (cluster 2) within porcine N1av (Figure 3a). The N1av sequences of recent German porcine isolates formed two sub-lineages but no correlation of these NA sub-lineages with the H1av clustering of the respective isolates was found.

Deeper fissures were evident in the phylogenetic tree of N2 sequences (Figure 3b), and recent German porcine isolates could be assigned to three porcine- and one human-derived, clearly distinct clusters. Three of these clusters had already been delineated earlier when the neuraminidase of H1pdmN2 reassortants was analysed (10). The N2 of the majority of non-reassorted H1N2 and H3N2 isolates, grouped within assigned porcine cluster 3 and was distinguished by amino acid substitutions L23F, T236I/V, I263V and L/I360T/I. Cluster 2 held most of the H1pdmN2 reassortant viruses although two H1avN2 reassortants (R369/09, R1931/11) which were conspicuously distinct in the HA1 sequence from all other H1av sequences also fell into this cluster. The N2 of porcine cluster 2 presented 12 unique amino exchanges (Y40C, P/I45S, N47V/I, I57K, E74K, K/R75T, P/T126L, H197Y, F205L, G/D/S313N, R/L338Q, E343Q). Only a few contemporary N2 porcine viruses from Germany segregated into porcine cluster 1. The N2 of three further H1pdmN2 reassortants was assigned to the human HxN2 cluster and showed a close relationship to human N2 viruses which had last been detected in the human population in mid 1990s. This N2 lineage has never been reported from European porcine influenza viruses; its origin remains uncertain.

Hemagglutinin and neuraminidase reassortant patterns and protein analysis
Reassortants between the HA and NA of the different viral subtypes were found for the H1 subtype only. Reassortants between H1N1pdm and porcine viral neuraminidase subtypes N1 and N2 have recently been described (9, 10). Viruses of the H1pdmN2 lineages continued to be in circulation and further evolved in late 2011 and early 2012 (e.g., R509/12, Figure 3a). In addition, reassortants between H1N2 HA and N2 derived from the porcine H3N2 lineage were observed: R757/10, R856/10, R74/11, and R21/12. Also, reassortants between the HA of the H1N1av-derived H1 lineage and NA N2 of different lineages were detected in altogether four cases in 2009 (R369/09), 2011 (R1931/11; R3508/11), and 2012 (R624/12). One reassortant between H1N2 HA and H1N1av-derived NA N1 (N1av) was detected in 2009 (R3394/09).

Two reassortants, R369/09 and R1931/11, revealed HA protein sequences which were comparatively distant from the average H1av HA of contemporaneously circulating viruses as signaled by long horizontal branches in Figure 3b. R1931/11 showed deletion of a lysine residue at position 147. In addition, this HA sequence possessed a set of an additional three predicted N-linked glycosylation sites (NGSs). Whereas all of the investigated H1av HAs share potential NGSs at amino acid positions 28 (3+), 40 (3+), 498 (+) and 557 (2+) (in brackets: positive score analyzed by NetNGlyc 1.0 Server), R1931/11 possessed further potential sites at positions 136 (NAT, 2+), 178 (NKS, +), and 211 (NHT, +).

The newly established neuraminidase sequences as listed in Table S1b were assessed for mutations (i) associated with decreased susceptibility, as confirmed by site-directed mutagenesis, to neuraminidase inhibitors oseltamivir (N1: H275Y, N295S; N2: R292K, E119V, N294S) and zanamivir (N1: Q136K, K150T; N2: R292K, E119A/D)), and (ii) for mutations observed during surveillance or following in vitro selection using oseltamivir (N1: I223R; N2: I222V). None of these resistance markers (28) were observed in the investigated sequences.

**Genotyping**

Full-length genomes were established for 15 porcine isolates. For three additional isolates one to two segments are missing (Table 2). Isolates were selected so as to represent a cross section of subtypes, HA/NA reassortment patterns, and year of isolation. PhyML analysis for “internal” segments (PB1, PB2, PA, NP, M, NS) included most closely related sequences of a BlastN2 search within the EpiFlu database as well as a random selection of additional sequences from Eurasian porcine and human isolates obtained since 2000. Genotyping results for each genome segment were obtained by use of the FluGenome tool (29) and are summarized in Table 2. The cassette of...
“internal” genome segments of Eurasian porcine IAV is composed of genotypes F, G, I, F, F, and 1E (PB2, PB1, PA, NP, M, NS) independent of the configuration of the HA and NA genome segments. Within each genotype two sister clades were phylogenetically distinguishable and are tentatively referred to as “1” and “2” (see supplemental Figures S2a-f). This is consistent with data of other German and European porcine IAV of the last ten years (sequences of 19 further viruses extracted from EpiFlu database; data not shown). The isolates R369/09 and R1931/11 exhibited PB2 and (R1931/11 only) NS sequence patterns which could not be classified by the genotyping tool. This confirms separate clustering of these isolates in the phylogenetic trees of the “internal segments” (supplemental Figure S2a-f). The H1pdm isolates of porcine origin including the H1pdmN2 reassortants (n=3) belonged to genotypes C, D, E, A, F, and 1A (PB2, PB1, PA, NP, M, NS) which is similar to other H1pdm viruses isolated from pigs in Europe and elsewhere (Table 2). Based on these results a total of three genome constellations or genogroups (Table 2) were distinguishable.

Characterization of “internal” gene segments

The majority of 15 non-H1pdm isolates analyzed here are, independently of their glycoprotein subtypes, true representatives of the Eurasian avian-derived cassette of “internal segments”. Within this cassette 19 unique amino acid exchanges have been identified which set them apart from classical swine H1N1 viruses (30). Deviations from this pattern were evident in the NS segment of four isolates (R248/09, R819/10, R1931/11, R535/12: W25Y, K66E and E,G227R).

PB1 and PA segments encode further proteins in different reading frames. Variations in the length of the PB1-F2 protein of European porcine influenza viruses have been identified (31). The majority of German isolates analyzed here (15 out of 18) encoded full length (90 aa) PB1-F2. However, two isolates (R1737/09 and R2807/09) appeared to produce truncated forms of 79 aa and, 87 aa, respectively. Two isolates (R369/09, R1931/11) carried the rare N66S mutation in PB1-F2 that has been associated with an influence on virulence (32). The porcine H1pdm viruses from swine in Germany displayed, similar to the human-derived ones, a PB1-F2 peptide of only 11 aa in length. The PA-X protein-coding region, accessed via ribosomal frameshifting, could be demonstrated in all porcine PA sequences established here (Table 2). All but two isolates carried the most common decanucleotide motif at the proposed frameshift site (UCC UUU CGU C, [33]). As analyzed by Shi et al. (34), the H1pdm viruses possess a stop codon at nucleotide 698 leading to a truncated PA-X (41 aa) whereas all other Eurasian porcine IAVs, including the ones analyzed here, should produce the full-length protein (61 aa).
Length variations were also observed in the NS1 protein. Full-length versions of 230 aa appear to be encoded by 11 out of 18 non-pdm SIV while the remaining six viruses for which NS sequences were established displayed a truncated NS1 protein of 217 aa, similar to some recent Italian isolates (35). These six truncated NS1 proteins were found in all three subtypes (each two times in H3N2, H1N2, and H1N1) and were isolated in 2009 and 2010. The NS1 length was not correlated to HA/NA subtypes. The sequence of the PDZ binding domain at the C-terminus of full length NS1 was the common G-P-E-V (36) in the majority of isolates. In addition, R-P-E-V (R24809) and R-P-K-V (R1931/11), respectively, occurred in one isolate each.

As expected from previous studies (37) amantadine resistance markers S31N and R77Q in the M2 protein were observed in all isolates analyzed here. A total of 16 non-pdm viruses also carried mutations L26I and V27A/T in the M2 protein. V27A/T also confers amantadine resistance.

Antigenic patterns of selected porcine influenza A viruses

Isolates representing the tips of the various sub-lineages discerned in the phylogenetic analyzes were selected for an antigenic analysis by HI using a set of post infection sera established recently in swine and ferrets. An overview of the results regarding the antigenic relationships within subtype H1 is presented in Figure 4. Titers obtained in HI assays (see supplemental Table S3a for absolute values) were transformed into a 2D antigenic map by agglomerative hierarchical clustering (HCE3.5). The heat map shown in Figure 4 was set at a similarity of 0.50 (50%) for the rows. Within the larger clusters of H1pdm, H1N2 and H1N1av further lineages can be distinguished: (i) Reassortant isolates of the H1pdmN2 subtype (R2035/11 and R509/12, green) can be discerned from “true” H1pdm antigens (dark red); (ii) - H1N2 viruses form a separate homogenous cluster (dark blue); (iii) the H1av viruses are antigenically split into two clusters; and (iv) the reassortant H1avN2 isolate R1931/11 is distinguishable phylogenetically and, less pronounced, also antigenically from other H1av isolates.

Although the H3 viruses formed two clusters in the HA phylogenetic tree (Figure 2d), a single homogenous group was found when typed with serum raised against an older (Bakum/99) and a very recent (R655/12) H3N2 isolate (see supplemental Table S3b). This finding is consistent with analyzes of the commonly accepted antigenic sites within H3 HA1 (38) where only marginal changes were observed: D48N [antigenic site C] in four sequences; I212V [antigenic site D] in two and I276T [antigenic site C] in 3 out of the 4 sequences of the small blue cluster in Figure 3d. No differences were seen regarding potential N-glycosylation sites (n=8).
Experimental animal infections

Two recent porcine isolates were selected for experimental infection studies in mini pigs. Isolate R2035/11 (H1pdmN2) belonged to the group of recently emerged reassortants between pandemic H1N1 and porcine HxN2 viruses. As shown in Figure 5 (upper panel) intranasal inoculation led to a productive infection with significant viral RNA excretion during the first week post infection. Virus was also transmitted to an in-contact pig which showed a similar but shifted course of viral RNA excretion. Mild respiratory symptoms such as sneezing and clear nasal discharge were observed for all animals around day 5 of infection. None of the pigs showed rectal temperatures exceeding 39.8°C.

Isolate R1931/11 (H1N2) represented a reassortant between avian-derived H1N1 and a porcine HxN2 virus. This isolate revealed several conspicuous genetic and phylogenetic features as described above and was also antigenically distinguishable from other H1av viruses (Figure 4). The lower panel of Figure 5 shows a similar course of nasal viral RNA excretion as described for R2035/11. Infection of the contact pig confirms productive infection leading to rapid transmission of virus. Clinical symptoms were similar to those of the R2035/11 group but were restricted to day 3 only. Likewise, fever was not noted.

Seroconversion by NP-specific blocking ELISA was evident from day 7 p.i. on for the R1931 group and from day 14 on also by homologous HI assay for both groups (data not shown). For unknown reasons no clear-cut seroconversion (days 7 and 14 p.i.) was evident for the R2035 group when using the NP-specific blocking ELISA.

Discussion

Porcine IAV infections in swine were frequently detected in studies A and B by virological means in a region of Germany with a high intensity of swine farming. Virus was found to circulate primarily in weaners affected with respiratory symptoms. This is in line with previous studies on the epidemiology of swine influenza viruses which have emphasized the importance of younger pigs as a motor of IAV infections in swine herds (39, 40). However, also the breeding sow population has been implicated as a reservoir of respiratory pathogens (41) and influenza viruses have been associated with abortion and other fertility disorders in sows (42, 43). In order to investigate such associations study B was conducted which focused on larger swine herds which reported fertility
disorders and records of influenza vaccination. The data are representative for swine holdings which keep sows but not for pure fattening holdings. Our results revealed that virologically detectable infection of older sows was a rare event (5.8% positive samples), yet circulating influenza virus was detected in weaners of such holdings (32.2% positive samples). As such, a direct link between influenza virus infections and reproduction failures in older sows could not be verified in this study. However, short febrile episodes caused by limited and short-lived virus replication, as observed during infection experiments (Figure 5), may have passed virologically unnoticed. Still, such episodes may have resulted in hormonal imbalances leading to abortion. Influenza viruses also play a significant role in the porcine respiratory disease complex (44), a difficult-to-define syndrome of multifactorial etiology in which complex and often synergistic interactions of different pathogens, including PRRSV and PCV-2, and environmental factors play a role (45-49). Interestingly, the questionnaire evaluation in study B showed that vaccination of sows against PRRSV and vaccination of weaners against PCV-2 appeared to have a substantial effect on reducing virologically detectable influenza virus infections in herds which had received continuous influenza vaccination of the sows. These findings prompt to further study co-infections or preceding infections with these viruses or other bacterial pathogens, which has not been specifically investigated in this study.

The passive surveillance carried out in the frame of study B confirmed year-round viral activity for endemic porcine influenza viruses (Figure 1). This is in contrast to the seasonal pattern of human influenza in temperate zones. Detection in swine of the human pandemic H1N1 virus of 2009 and reassortants thereof outside the human influenza season (May-December) indicated independent circulation of these viruses in the swine population under study (Table 1). Another study which involved five European countries excluding Germany also reported year-round influenza virus activity in swine herds with peaks during December and May (49). This is corroborated by serological data collected in a three years period in four European countries showing only marginal seasonal variation of seroprevalences (50). All-year influenza virus activity in swine herds entails a continuing risk of transmission of porcine influenza viruses from swine to man. It is of importance to note that three infections with influenza viruses of humans in Germany which occurred outside the usual influenza season in 2010 and 2011 were in fact caused by endemic porcine influenza viruses (51).

The characterization of porcine IAV revealed presence of the three standard European subtypes H1N1, H1N2 and H3N2. The use of nasal swab samples obtained from acutely infected
pigs was advantageous over using lung tissue samples from terminally diseased swine for subtype-
specific characterization (77.9% vs. 30.2% of fully subtyped positive samples; Table 1, upper panel)
but not for generic detection of swine influenza viruses (21.4% vs. 24.4%). This was largely due to
the fact that up to ten nasal swab samples of a herd in contrast to one or two tissue samples were
available for selection of those samples with higher contents of viral RNA for subtyping. The
principal quality of RNA from swabs versus lung tissues, however, did not cause this difference in
successful subtyping. Viruses of the avian-derived H1N1 lineage made up almost two thirds of the
fully subtyped samples. H1N2 and H3N2 viruses were detected at grossly lower prevalences (17.6
and 11.1%, respectively). The dominance of H1av-like viruses is in line with earlier reports from
Germany (6, 52) and other European countries (49). Viruses of the human pandemic H1N1/2009
virus were repeatedly introduced from the human population into swine herds in Germany since
2009 (9). Considering data which demonstrated high susceptibility of swine for this lineage and
ease of transmission between pigs (18, 40) it seems odd that only two herds with H1N1pdm
infections have been found during the surveillance period (Table 1). No immunization against this
lineage had been carried out during the study period. However, reassortants of H1pdm with
porcine HxN2 viruses emerged since May 2011 in this region (10) and continued to circulate until
the end of the study period when, finally, 11 herds with H1pdmN2 infections were found. It is
tempting to speculate that H1pdmN2 viruses have evolved a selective advantage over the
H1N1pdm lineage and are therefore spreading preferentially. In countries where such reassortants
have not emerged, e.g., the U.K., H1N1pdm has obviously gained larger grounds (40). The
hemagglutinin of most isolates of the H1pdmN2 reassortant viruses clustered separately from 2009
H1N1pdm in a new clade. Both the HA and the NA of these viruses are characterized by a large set
of amino acid substitutions which separate them from their ancestral sequences. As an optimal
balance between the functions of the counteracting HA and NA glycoproteins is required for fitness
of the virus, it may be speculated that the recorded substitutions represent the current result of
mutual adaptations to the new partner protein.

The phylogenetic analyzes of HA1 and NA sequences gave further insight into the diversity of
circulating porcine influenza viruses: Recent H1N2 and H3N2 HA1 sequences formed
monophyletic clades which were temporally (H3N2) or geographically (H1N2) separated from their
nearest neighbors in the trees (Figure 2d and supplemental Figure S2). The H1N2 viruses in
particular seem to have undergone separate evolution in different European countries. This seems
to point towards transmission chains that do not cross political borders in the common European
market. In this respect studies on networks in swine trade in several European countries point out
that the vast majority of trading actions is compartmentalized and restricted to few, spatially
clustered communities with rare contacts outside these trading pools (53, 54). This might explain
to some extent geographic restriction of porcine influenza virus evolution although it is not clear
why this should affect predominantly viruses of subtype H1N2.

Complex branching patterns were evident for the HA1 of avian-derived H1. At least for sub-
lineages were discernible on basis of their nucleotide sequences and some could be distinguished
by unique amino acid substitution patterns as well. Similar diverging trends have also been noticed
for the classical porcine H1N1 virus in North America (55). In the current study one group of
related viruses (R369/09, R848/11, R1931/11, R3310/11) were placed on unusually long horizontal
branches and showed a particular accumulation of amino acid substitutions. These affected also
the pattern of potential N-linked glycosylation sites which is known to have an impact on
antigenicity and immunogenicity (56, 57). N1 sequences of porcine influenza viruses from Germany
fell into three lineages all of which were derived from the same avian precursor. The origin of N2
sequences was assigned to four lineages. H1pdmN2 viruses had derived their N2 from three of
these lineages (10). One lineage was derived from precursor viruses prevalent in the human
population during mid 1990s. No other porcine viruses carrying a neuraminidase of this lineage
have been described so far. Since this lineage also seems extinct in the human population the
reservoir of this neuraminidase remains obscure.

Nine reassortment patterns involving surface glycoprotein encoding segments of the endemic
H1N1av, H1N2 and H3N2 Eurasian porcine subtypes were detected in this study within a
surveillance period of 19 months. This is a rate of 10.2% given a set of 88 fully subtyped viruses.
Since fewer NA than HA sequences have been generated in this study this may still be an
underrepresentation of reassorted viruses actually in circulation. Yet, the proportion of reassorted
viruses observed here is higher and their diversity wider than recently estimated for Eurasian swine
influenza viruses (58) and recently shown in field data by Kyriakis et al. (59) who reported a
reassortment rate of 3% during a 3 years surveillance period in samples from five European
countries excluding Germany. The comparatively frequent incident of HA/NA reassortments is also
reflected by the fact that in five cases infections with two different subtypes have been detected at
the same time in the same herd but in different animals (Table 1 “double”).

In contrast to the variability of HA and NA and their frequent reassortments the polymerase
complex but also the remaining “internal” gene segments of recent porcine IAVs from Germany
seem to be comparatively stable with respect to their sequence and their gene constellations (Table 2, supplemental Figure 2). Three genogroups were distinguished in this study based on the limited number of 18 viruses that were fully sequenced. The only segment which showed reassortment apart from HA and NA was NS in two cases. This finding is in line with recent analyzes of Eurasian swine influenza virus sequences indicating that the current set of avian-derived Eurasian lineage “internal” gene segments (genogroup 1, Table 2), and here especially the polymerase complex, seems to form a stable constellation similar to the TRIG cassette of North American porcine viruses (58). In contrast, however, to the situation in the U.S. where frequent reassortants of “internal segments” between the pandemic H1N1pdm and American endemic porcine viruses have been found (59) no such reassortants were detected in the present study.

Genogroup 3 which represents the standard set of segments of H1pdm viruses was retained also in the H1pdmN2 reassortant viruses analyzed here. Genogroup 2 (R369/09 and R1931/11), however, appears to typify another gene constellation which has not been described before. These viruses, together with R3310/11 and R848/11, form a loose group of isolates with an H1av HA and either N1av or N2hu NA. In addition, in one of these viruses (R1931/11) aa147 in the HA protein was deleted and aberrant patterns of potential NGs were expressed. The NS1 gene segment of this isolate harboured a variant PDV domain (RPKV) and could not be typed with the FluGenome genotyping tool; its PB1-F2 segment expressed the rare N66S substitution.

The different character of isolate R1931/11 is also reflected in its distinct antigenic properties which were demonstrated by HI using a panel of polyclonal post-infection sera from swine and ferrets. The normalized cross HI titer panel depicted as a heat map and a corresponding dendrogram in Figure 5 allows the distinction of the standard subtype H1 viruses into avian-derived H1av, pandemic H1pdm and H1[N2]. While the H1[N2] isolates formed a homogenous block, lineages were discernible among the H1av and the H1pdm viruses. In the latter lineage H1pdmN2 reassortants were distinguishable from “standard” H1pdm circulating in swine but also in humans (R26/11) in Germany. Within the H1av lineage two antigenically distinct groups were evident. R1931/11 is associated with one of these H1av groups but forms a separate branch.

Deletion ΔK147 (H1 numbering) detected solely in this isolate exactly matches position K134 (H3 numbering) in human seasonal H1 viruses (Table 3); deletion of K134 has been shown to have caused significant antigenic drift in those strains (60). The segregation of R1931/11 suggested on basis of the HI antigenic properties was fully mirrored by a phylogenetic analysis of the deduced HA1 amino acid sequence of these viruses (Figure 4, left tree). The epidemiological relevance of
diverging antigenic trends in H1av and H1pdm remains to be analyzed. This would also include vaccination-challenge experiments to explore cross-protective vs. neutralization-escape tendencies of the emerging lineages.

Two viruses from lineages with conspicuous genetic, phylogenetic and antigenic alterations, when compared to the standard European subtypes, where selected for infection experiments in minipigs. Both the H1pdmN2 isolate R2035/11 and the aberrant H1avN2 reassortant R1931/11 replicated productively in pigs following intranasal inoculation and were readily transmitted to an in-contact sentinel. This indicates potential of these viruses for further spread in the swine population. The very mild clinical course observed should not be over-interpreted as minipigs from a closed breeding nucleus may react differently and show milder clinical symptoms compared to standard pig lineages: E.g., minipigs did not show clinical symptoms when challenged with the California/04 H1N1pdm isolate (61) which, in contrast, produced moderate symptoms similar to endemic porcine influenza in standard pigs (62, 63). Moreover, the minipigs were 13 weeks of age when inoculated and the increased age, compared to younger weanling pigs, may be more explanatory for the mild clinical signs.

In summary, we have shown frequent, widespread and year-round circulation of at least four stable lineages (H1N1, H1N2, H3N2, H1pdmN2) of porcine influenza viruses and focal presence of further reassortants between these lineages in a region of Northwestern Germany which holds 55% of the country’s pig population. Trends for on-going diversification both at the phylogenetic and the antigenic levels were demonstrated for the H1N1av lineage and its reassortants which still dominated the epidemiological situation. In addition, continuing diverging evolution was also noticed for the recently emerged H1pdmN2 lineage. These viruses carry seven segments of the human pandemic H1N1 2009 virus including an antigenically markedly altered hemagglutinin and different variants of porcine- or human-derived subtype N2 neuraminidase. The zoonotic potential of this lineage remains to be determined. A tight control of influenza virus infections by improved herd management, highly efficacious vaccines and effective herd vaccination regimes is highly desirable with respect to health and productive potential of swine herds. Controlled regular influenza virus surveillance in swine populations is a pivotal prerequisite of this goal. In line with the One Health concept reduced circulation of influenza viruses in pigs also means lowered risks of the formation of new reassortant viruses with unknown zoonotic potential and their transmission to humans.
Acknowledgments

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We acknowledge the authors, originating and submitting laboratories of the sequences from GISAID’s EpiFlu™ Database on which this research is based. The list is detailed in supplemental Table S4).
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Legend to figures

Figure 1. Frequency of porcine influenza virus infection in selected swine herds (upper panel) and individual animals (lower panel) sampled in Northwest Germany, March 2011 - March 2012. Black bars– absolute numbers of herds/animals sampled (left y-axis); shaded – absolute numbers of herds/animals which tested positive (right y-axis), grey – percentage of herds/animals which tested positive (right y-axis).

Figure 2. Phylogenetic analysis in a Bayesian framework of the hemagglutinin gene HA1 fragment (A – subtype H1pdm, B – subtype H1av, C – subtype H1(N2), D – subtype H3) of porcine influenza A viruses detected in selected swine herds in the Northwest of Germany, 2009 - 2011. Trees are drawn to scale as indicated by the scale bar. A red dot on a branch depicts sequences that were established in the frame of this study. Clustering information is given by colored branches and/or by specific assignments to the right of the trees. The screened tree shown in Figures 2a-c represent a PhyML analysis of an enlarged set of porcine subtype H1 sequences to allow an overview of phylogenetic distances within this subtype. Sequences established in the frame of this study are shown with specific registration number and year of isolation. EpiFlu database accession numbers can be retrieved from supplemental Table S1. Further sequences have been extracted from GenBank or the EpiFlu databases and their accession numbers are indicated in the trees. Details of the phylogenetic analysis are given in the Methods section. Red color for subtype characterization indicates presence of a reassorted neuraminidase segment.

Figure 3. Phylogenetic analysis in a Bayesian framework of the neuraminidase gene (A – subtype N1, B – subtype N2) of porcine influenza A viruses detected in selected swine herds in the Northwest of Germany, 2009 - 2011. Trees are drawn to scale as indicated by the scale bar. A red dot on a branch depicts sequences that were established in the frame of this study. Clustering information is given by colored branches and/or by specific assignments to the right of the trees. Sequences established in the frame of this study are shown with specific registration number and year of isolation. EpiFlu database accession numbers can be retrieved from supplemental Table S1. Further sequences have been extracted from GenBank or the EpiFlu databases and their accession numbers are indicated in the trees. Details of the phylogenetic analysis are given in the Methods section.
Figure 4. Clustering of porcine influenza virus isolates of subtype H1 by two-dimensional antigenic hierarchical clustering based on HI titres. The heat map shows three-color-coded (see scale bar) normalized similarity values (light green: lowest, black: intermediate, and bright red: highest similarity). The dendrogram to the right is drawn in scale according to the antigenic hierarchical clustering, and the viruses in this dendrogram each line up with a row in the heat map. The sera used in HI assays at the top of the heat map have been given colors that match that of the viruses in the dendrogram to the right. The dendrogram to the left is based on a phylogenetic analysis (PhyML) of amino acid sequences of the HA1 protein of the same viruses used in cross HI assays.

Figure 5. Experimental infections of mini pigs. Course of the infection with reassortant virus R2035/11 (H1pdmN2) and R1931/11 (H1avN2), respectively, as mirrored by presence of viral RNA in nasal swabs detected by M-specific RT-qPCR (Cq values). Filled square and ellipse – animals inoculated intranasally; open lozenge – sentinel animal, placed with the inoculated ones on day 1 p.i.
Table 1. Subtype characterization of porcine influenza viruses from selected swine holdings in Northwest Germany, 2009-2012.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Study A Months (2009 - 2010)</th>
<th>Study B Months (2011 - 2012)</th>
<th>Results</th>
<th>%</th>
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<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
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<td>Sum 1</td>
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Viruses of study A were detected in lung tissues of weaners with severe respiratory disease. Study B samples consisted of nasal swabs of sows, piglets or weaners from large holdings with fertility problems. Sum 1 refers to all fully subtyped viruses (percentages in brackets), sum 2 includes also partially (e.g., H1Nx) or non-subtyped viruses (HxNx). Each sample represents one holding. In five cases double infections with H1N1/H1N2 (a) or H1N1/H3N2 (b) were detected. In these cases both viruses were added.
<table>
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<th>NA</th>
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<th>PB2</th>
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</tr>
</tbody>
</table>

The genotypes were assigned according to results of FluGenome genotyping tool and a series of PhyML analyses of the complete open reading frame of each of the internal genome segments (see supplemental Figures S2a-f). Numbers “1” and “2” specifying some of the genotypes refer to different sister clades within that respective genotype as shown in the supplemental figures. The column “genogroup” represents a summary of the genotype constellation of the internal segments.

*All NS sequences clustered with allele “A”. ND – no data available.

? - No unambiguous classification possible using the FluGenome genotyping tool (25).
Table 3. Deletion of lysine residue K147 (H1 numbering) in the HA1 fragment of R1931/11 (H1avN2) matches deletion K134 (H3 numbering) in human seasonal H1 viruses known to have caused antigenic drift (McDonald et al., 2007).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Beijing/262/95 (H1N1)</td>
<td>126-S WP NT V T – GV T A SC</td>
</tr>
<tr>
<td>A/Shenzhen/227/95 (H1N1)</td>
<td>126-S WP NT VTKGVTASC</td>
</tr>
<tr>
<td>A/Swine/Germany/R1931/11 (H1avN2)</td>
<td>139-S WP DHKT T – G TT G SC</td>
</tr>
<tr>
<td>A/Swine/Germany/R655/12 (H1N1av)</td>
<td>139-S WP HETTKGSTVAC</td>
</tr>
</tbody>
</table>

Bold-face lettering indicates the deletion site.
Figures

Figure 1.
Figure 2a.
Figure 2c.
Figure 2d.
Figure 3b.

Porcine H9N2
Human HxN2 strains

Porcine HxN2 strains, cluster 1

Porcine HxN2 strains, cluster 2

Porcine HxN2 strains, cluster 3