Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany

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The incursion of the human pandemic influenza A virus H1N1 (2009) (H1N1 pdm) into pig populations and its ongoing co-circulation with endemic swine influenza viruses (SIVs) has yielded distinct human–porcine reassortant virus lineages. The haemagglutinin (HA) gene of H1N1 pdm was detected in 41 influenza virus-positive samples from seven swine herds in northwest Germany in 2011. Eight of these samples yielded virus that carried SIV-derived neuraminidase N2 of three different porcine lineages in an H1N1 pdm backbone. The HA sequences of these viruses clustered in two distinct groups and were distinguishable from human and other porcine H1 pdm by a unique set of eight non-synonymous mutations. In contrast to the human population, where H1N1 pdm replaced seasonal H1N1, this virus seems to co-circulate and interact more intensely with endemic SIV lineages, giving rise to reassortants with as-yet-unknown biological properties and undetermined risks for public health.

The concept of pigs as mixing vessels in the influenza A virus ecosystem has been rejuvenated since the emergence of the human pandemic H1N1 virus in 2009 (Irvine & Brown, 2009). Due to their broad repertoire of influenza virus receptors, swine can be infected by influenza A viruses of porcine but also of avian and human origin (Van Poucke et al., 2010). Vice versa, viruses of different swine-adapted lineages (classical swine, North American triple reassortant, Eurasian avian-like lineages) can be transmitted back to poultry (Starick et al., 2011a) and to humans (Myers et al., 2007; Shinde et al., 2009; WHO, 2011). The emergence and spread of the pandemic H1N1 influenza A virus in 2009 (H1N1 pdm) were perpetuated entirely by the human population. However, soon after the virus started to spread globally in humans, its introduction by infected humans into pig holdings was noticed in several countries (Ducatez et al., 2011). A high susceptibility of pigs to H1N1 pdm was confirmed by transmission experiments (Lange et al., 2009; Brookes et al., 2010), which also showed that infection was associated with clinically apparent disease and efficient pig-to-pig transmission.

This immediately raised concerns regarding the generation of reassortants between H1N1 pdm and viruses of currently circulating swine influenza virus (SIV) lineages. Such reassortants, detected in several countries, may express altered phenotypic characteristics and pose an additional risk for the human population. A first report from Hong Kong described a reassortant containing the North American triple reassortant internal gene cassette, a Eurasian swine lineage haemagglutinin (HA)-encoding gene segment and the neuraminidase (NA)-encoding segment derived from H1N1 pdm (Vijaykrishna et al., 2010). Subsequently, additional reassortant genotypes were found in Italy (Moreno et al., 2011), Germany (Starick et al., 2011b), China (Zhu et al., 2011; Fan et al., 2012), Thailand (Kitikoon et al., 2011), the UK (Howard et al., 2011), Argentina (Pereda et al., 2011) and Korea (Han et al., 2012). In the USA, nine reassortant viruses, representing seven genotypes, were detected in 2009 and 2010 (Ducatez et al., 2011). Eight of them displayed HA and NA genes of endemic SIV and carried the matrix (M) and various other gene segments of H1N1 pdm. Further reassortant viruses were reported most recently from the USA (Liu et al., 2012; Ali et al., 2012).
Recently, viruses with surface protein-encoding genes of subtype H3N2 from North American swine viruses and all remaining genes from H1N1 pdm were reported from Canada (Tremblay et al., 2011). Finally, in the USA, several human cases of infection with reassortant influenza virus that carried the H1N1 pdm M gene in the backbone of the circulating swine H3N2 virus were reported, mainly in children (WHO, 2011). So far, little is known about the phenotypic characteristics of these reassortants and whether they will expand in porcine and/or human populations.

The first detection of H1N1 pdm in a pig holding in Germany dates back to December 2009, and, in May 2010, a first reassortant H1N1 pdm comprising seven segments of the H1N1 pdm genome and SIV NA of subtype N1 was isolated from pigs in the northern part of Germany (Starick et al., 2011b). In order to follow the fate of these viruses, a regionally limited surveillance has been carried out in pigs in north-west Germany since March 2011. Up to December 2011, a total of 2078 swine nasal swab samples from 292 holdings had been investigated by real-time RT-PCR specific for an M gene fragment. A fraction of 502 samples (24.2 %) from 119 holdings (40.8 %) tested positive for influenza A virus RNA. Of these, 41 samples (8.2 % of the positive samples) from seven holdings (5.9 % of the positive holdings) were found to be positive for the HA of H1N1 pdm by HA subtype-specific RT-PCR (Hoffmann et al., 2010). Thirteen representatives of these viruses were characterized molecularly in greater detail; five were identified as H1N1 pdm viruses with no evidence for reassortment [all genome segments were of pandemic origin using RT-PCR typing as described by Ducatez et al. (2010)]. The remaining eight samples from five holdings, however, harboured NA of subtype N2 in the background of seven gene segments of the H1N1 pdm virus. These N2 reassortants are designated H1pdmN2 here.

Full-length HA gene sequences of H1N1 pdm viruses isolated from different animal species (swine, cat, turkey, ferret) in Germany and elsewhere were analysed phylogenetically and compared with human isolates from Germany and the Netherlands (Fig. 1a). A high degree of sequence conservation was present among the selected H1pdm HA gene segments. However, sequences of H1pdmN2 reassortants of pigs from Germany formed two distinguishable clusters (A, B), as shown by high posterior probability values in the Bayesian phylogenetic analysis. These viruses also differ from other, previously described reassortants that carried the HA H1 pdm and a reassortant NA, e.g. A/swine/Germany/R708/10 and A/swine/Thailand/CU-SA43/2010 (both H1pdmN1sIV) or A/swine/Italy/116114/2010 and A/swine/Korea/494/2010 (both H1pdmN2). Cluster A H1pdmN2 reassortants (R75, R1048, R1050, R2035, R2356 and R2385) are distinguished by a unique set of mutations comprising an exchange of 16 nt and 8 aa across the gene (Table 1). Seven of the eight non-synonymous mutations clustered in the HA1 part of the protein (K159R, G172E, I183V, S200P, S202N, D204S, V338I) and one in the HA2 fragment (V466I). Four of the amino acid exchanges were located in or in the proximate neighbourhood of putative antigenic sites that were termed Sa, Sb and Ca by Igarashi et al. (2010). Sites Sa and Sb have been characterized as targets of neutralizing antibodies (Igarashi et al., 2010) (Table 1). Three of the mutated sites (K159R, S200P and D204S) have previously been described to be involved in receptor glycan contacts of H1 pdm HA (Soundararajan et al., 2009). The HA gene of H1pdmN2 reassortants of cluster B (R2432 and R2448, which originated from the same holding) did not share these mutations. However, four identical potential N-glycosylation sites were identified for viruses of both cluster A and B (NetNGlyc server score $\geq + +$, http://www.cbs.dtu.dk/services/NetNGlyc; amino acid positions 28, 40, 304 and 557). Cluster A viruses harboured an additional putative N-glycosylation site due to mutations S202N and D204S (N202ASQ, score +). This site is missing from cluster B viruses and other H1pdmN2 viruses from Italy and Korea and is also lacking in further pig-derived H1pdmN1 from Germany with either pandemic N1 or SIV-derived N1. Analysis for selection pressure on the HA1 part of the HA protein by single likelihood ancestor counting, fixed and internal fixed effects likelihood, and mixed effects model of evolution concordantly gave evidence for positive selection acting at site 202 ($P<0.05$ in at least three methods; analysis carried out using the HyPhy software suite at www.datamonkey.org). Position 202 is part of the neutralization-relevant site Sb; all H1pdmN2 viruses of cluster A carried N at this position, whilst all other H1pdm proteins analysed carried S or T.

Phylogenetic analysis of the NA genes of the H1pdmN2 reassortants revealed that they originated from three different, clearly distinct lineages (Fig. 1b). Viruses R75, R1048, R2035, R2356 and R2385 carried an N2 segment that had previously been described in novel reassortant swine viruses of subtype H1N2 (Eurasian avian type) from Sweden and Italy (here referred to as NA cluster 1); these viruses had picked up the NA from porcine Eurasian H3N2 viruses (Bálint et al., 2009; Moreno et al., 2012). Virus R1050 was sampled in the same holding and at the same time as R1048 but bore, in contrast, an N2 segment from the authentic Eurasian porcine H3N2 lineage (NA cluster 2). The remaining two H1pdmN2 viruses (R2432 and R2448) carried an NA that was related closely to an older H3N2 virus of human origin (NA cluster 3). This virus circulated in 1995 and 1996 in the Eastern parts of the USA and was also isolated from humans in Italy. The N2 segment of this lineage has never been reported in a porcine influenza virus and has not been detected in humans since 1996; at least, no sequences thereof have been deposited in the EpiFlu or IRD databases. Selection pressure analysis, as described above, identified codons 30 and 371 (four of four methods, $P<0.05$), as well as sites 40, 128 and 142 (three of four methods, $P<0.05$), as being under positive selection. Furthermore, NA cluster 3 viruses possessed an additional (fourth) potential N-glycosylation site at position 86 (NWSK) compared with N2 sequences of clusters 1 and 2.
Fig. 1. Phylogenetic analysis of HA (a) and NA (b) gene segments of H1pdmN2 reassortant influenza A viruses detected in swine in north-west Germany in 2011. For comparison, sequences representing the HA and NA ORFs of other related influenza A viruses from different host species were selected from the EpiFlu database (accession numbers indicated) and aligned using the MAFFT algorithm (http://mafft.cbrc.jp/alignment/server/). Sequences generated in the frame of this study are displayed in bold type. JModeltest (http://darwin.uvigo.es/software/jmodeltest_server.html) was used to choose the most appropriate mutation model (GTR+I+Γ). Phylogenetic relationships were estimated in a Bayesian framework [software suite MrBayes, implemented in the Phylemon public server (http://phylemon.bioinfo.cipf.es/)]. The unrooted tree was drawn to scale (see scale bars) by use of the FigTree software (http://tree.bio.ed.ac.uk/software/figtree/) and edited further by Inkscape (http://inkscape.org/). Phylogenetically distinguishable clusters as shown by Bayesian posterior probabilities (see numbers at nodes) are referenced by letters (A and B; HA) or numbers (1–3; NA).
Antigenic relationships of cluster A and B viruses were assayed by haemagglutination inhibition (HI). Five sera raised against representative viruses of endemic Eurasian SIV lineages, H1N1pdm, and a representative of the H1pdmN2 reassortant viruses were used (Table 2): the serum raised against authentic human-origin H1N1pdm reacted with all porcine-derived isolates carrying an H1pdm HA gene. Serum raised in pigs against H1pdmN2 R 2035/2011, in contrast, reacted solely with H1pdmN2 reassortants, but not with other H1pdm viruses of either porcine or human origin. The relevance of this finding is compromised, however, by the comparatively low homologous titre of the H1pdmN2-specific serum.

Infections with H1N1 pdm-related viruses in the pig population of north-west Germany still played a minor role in the influenza epidemiology in that region until the end of 2011 (5.9 and 8.2% of positive holdings or samples, respectively). This geographical area holds the majority of the swine population of Germany at a high population density; prevalences of H1N1 pdm-like viruses in swine may differ in other regions of the country. In light of the total number of swine holdings infected with H1N1 pdm-like viruses (n=7), the number of infections with reassortant H1N1 pdm viruses carrying a porcine- or human-derived N2 NA (n=5) is high. Representatives of at least three distinct reassortment events with viruses of three

### Table 1. Overview of non-synonymous mutations in the HA gene of H1pdmN2 reassortant swine influenza viruses (SIV) of phyloclusters A and B detected in swine in north-west Germany in 2011

Amino acids are shown by their single-letter code; bold type indicates a mutation compared with the H1pdm consensus sequence; italics indicate new putative N-glycosylation sites.

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<tr>
<th>Virus Subtype</th>
<th>Amino acid position</th>
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<tr>
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<td>159</td>
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<td></td>
<td>Ca*</td>
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<tr>
<td>A/California/7/2009†</td>
<td></td>
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<tr>
<td>A/swine/Italy/116114/2010‡</td>
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<tr>
<td>A/swine/Germany/R2035/2011 (representing cluster A)§</td>
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<td>A/swine/Germany/R2448/2011 (representing cluster B)§</td>
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*Antigenic sites according to Igarashi et al. (2010); Sa and Sb are neutralization-relevant sites.
†Representative human isolate of pandemic H1N1/2009.
‡Reassortant H1 pdm virus carrying NA of subtype N1 derived from an avian-derived Eurasian porcine influenza virus.
§Representative viruses of HA phyloclusters A and B characterized in this study.

### Table 2. Antigenic relationships of H1N1 pdm reassortants investigated by HI assay

log$_2$ HI titres are shown; homologous antigen–serum pairs are shown in bold type. Antisera were raised in pigs against the following viruses: H1N1, A/swine/Germany/Belzig/2/2001; H1N1 pdm, A/Germany/Bavaria/74/2009; H1N2, A/swine/Germany/Bakum/183/2000; H3N2, A/swine/Germany/Belzig/54/2001; H1pdmN1, A/swine/Germany/R2035/2011.
different NA N2 lineages have been detected. Reassortants of HA cluster A/NA cluster 1 were isolated over 7 months and from different holdings in north-west Germany, indicating prolonged temporal and at least limited regional spread. The prominent changes in the HA gene of HA cluster A viruses set them apart from any porcine-derived bona fide H1N1 pdm viruses. Whilst positive selection did not seem to play a major role in the development of mutations in the HA1 protein fragment, it is tempting to speculate that the observed eight unique non-synonymous mutations were driven in response to the acquisition of the porcine N2 cluster 1 lineage NA. Nevertheless, the H1pdmN2 reassortants seem to be antigenically distinguishable from other isolates carrying an HA gene of H1pdm origin when using H1pdmN2 homologous serum. Further investigations of the biological characteristics of these viruses are necessary to determine their putative fitness gains and selection advantages. Other H1pdmN2 reassortants (HA cluster A/NA cluster 2, and HA cluster B/NA cluster 3) were detected in single holdings only.

In summary, co-circulation in the swine population in north-west Germany of H1N1 pdm viruses with endemic porcine influenza viruses gave rise to a set of reassortants with the NA of several SIV lineages. These reassortants (H1pdmN2) carry distinct mutations in the HA gene that distinguish them from standard H1N1 pdm viruses. The biological properties of these H1pdmN2 viruses, including their potential to be retransmitted to humans or to escape vaccine-induced immunity, need to be determined. Integrated and intensified influenza surveillance in pigs is required to monitor virus evolution with a public-health perspective. This demand is also emphasized by the (re)discovery in H1pdmN2 reassortants of an NA N2 lineage that had surfaced in human strains in the mid-1990s, but was not detected in the meantime.

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