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Phylogenetic analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three separate introductions of H5N1 virus

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Abstract

In spring 2006, highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 was detected in Germany in 343 dead wild birds, as well as in a black swan (*Cygnus atratus*) kept in a zoo, three stray cats, one stone marten (*Martes foina*), and in a single turkey farm. In 2007 (June-July) the virus reoccurred in 96 wild birds at six geographically separate locations in the Southeast of Germany. In addition, a backyard mixed duck and goose holding was affected. Real-time RT-PCR (Hoffmann et al., 2007) and nucleotide sequencing confirmed that these H5-viruses belonged to the Qinghai lineage of HPAIV H5N1 (clade 2.2). For a more detailed analysis, the hemagglutinin and neuraminidase genes of 27 selected German H5N1 viruses isolated 2006 or 2007 and originating from different regions and animal species were sequenced and analysed phylogenetically. As a result, three closely related but distinguishable H5N1 subclades could be defined: In 2006 a 'Northern type' (subclade 2.2.2), representing virus isolates from the German federal states Mecklenburg-Western Pomerania, Schleswig-Holstein, Brandenburg, and Lower Saxony, and a 'Southern type' (subclade 2.2.1) from Baden-Württemberg and Bavaria were detected. Interestingly, representatives of both types were present in Central Germany and caused the outbreak in turkeys (subclade 2,2,2) and a case in a tufted duck (*Aythya fuligula*) (subclade 2.2.1) in Saxony. Furthermore, one isolate from the South of Germany was identified as 2.2.2 and vice versa a 2.2.1-like isolate was found in Northern Germany. H5N1 viruses isolated in 2007 belonged to a third type (subclade 2.2.3) which was not detected in 2006. Our data suggest the introduction of three distinct H5N1 variants into the wild bird population of Germany. The source of these viruses and the exact time of introduction remain obscure. Based on the identification of closely related H5N1 viruses from Southern and Central Russia, a recent introduction via wild birds on winter escape from these regions, early in 2006 constitutes the most likely scenario for the 2006 outbreaks. The viruses detected in 2007 most likely represent another new incursion from an as yet unknown source.

Keywords

Avian influenza, H5N1, highly pathogenic, phylogenetic analysis, molecular epidemiology

Introduction

Influenza A viruses are maintained as biotypes of low pathogenicity (LPAIV) in their reservoir hosts, aquatic wild birds. However, upon transmission to gallinaceous poultry

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viruses of the subtypes H5 and H7 may mutate to highly pathogenic (HPAIV) strains by insertional mutation at a sequence of the HA gene encoding an endoproteolytic cleavage site (Swayne and Suarez, 2000). Thus, insertion of basic amino acid residues (arginine, R, and lysine, K) can give rise to a polybasic cleavage site accessible to ubiquitous proprotein convertases (Senne et al., 2006). In contrast to LPAIVs with a monobasic HA cleavage site, the polybasic cleavage site renders these avian influenza viruses competent for efficient systemic replication in their avian hosts. HPAIVs cause severe mortality, particularly in gallinaceous birds, and lead to severe losses in the poultry industry (Capua and Alexander, 2006). Twenty-four HPAI outbreaks among poultry have been recorded since the 1950s with variable severity and duration (Harder and Werner, 2006). All HPAI outbreaks were caused by unique viruses of either H5 or H7 subtype which apparently arise *de novo* from precursor viruses of low pathogenicity circulating in local wild bird populations.

In 1996, an H5N1 HPAIV (subtype Asia) was detected in geese in Southern China. Since then this virus established endemic infections in poultry, mainly ducks and geese, in many Southeast Asian countries (Li et al., 2004; Smith et al., 2006b). The geographical extent and duration of this outbreak as well as the number of poultry involved is unprecedented. Moreover, the pronounced zooanthroponotic properties of this virus are unusual in comparison with previous HPAI outbreaks (WHO, 2005). More than 300 documented human infections resulting in the death of more than 190 patients sparked fears of a new influenza pandemic caused by this virus (WHO, 2007). The transmission of HPAIV H5N1 of the Asian lineage from poultry back to wild birds led to previously unheard of major epidemics of HPAI among susceptible geese, gulls and other waterbird species in April 2005 at Lake Qinghai, North-West China (Chen et al., 2006). This event marked the start of an accelerated spread of H5N1 HPAIV through Central Asia into the Black Sea region and further westward into Central Europe. Phylogenetic studies have identified to date nine major clades of H5N1 HPAIV of Asian origin (WHO/FAO/OIE H5N1 Evolution Working Group, 2007). Clade 2 appears to be most diversified, and members of subclade 2.2 have been shown responsible for the westward spread of H5N1 since 2005 (Chen et al., 2006). Meanwhile, within subclade 2.2 three further subtypes have been distinguished and were designated with respect to their geographic origin European-Middle East-African (EMA-) 1 to 3 (Salzberg et al., 2007).

The first occurrence of H5N1 HPAIV in Europe was reported from Croatia and Romania in summer and fall 2005. A resurgence of the infection became apparent almost simultaneously in January/February 2006 in several geographically widely spaced locations along a line from Southern Sweden to Sicily. This included Germany where H5N1 HPAIV was reported in

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February 2006 when wild birds, predominantly swans (*Cygnus sp.),* were affected. Initially, the virus appeared to spread from a focus at the Baltic Sea coast on the Island of Ruegen. Within two weeks, however, the virus had been detected in seven Federal States of Germany, involving more than twenty different wild bird species. Ultimately, 343 H5N1 HPAIV infected wild birds were detected in Germany, in 2006, during extensive virological monitoring of wild bird carcasses of which more than 60.000 were analyzed. In addition, the virus was recovered from three stray cats and a stone marten (*Martes foina*) found moribund or dead on the Island of Ruegen at the height of the outbreaks among wild birds in March. Furthermore, in April, just after the peak of the outbreaks in wild birds, a mixed poultry holding in Saxony was found to be infected with HPAIV H5N1 of Asian lineage. Due to rapid adequate disease management efforts, the infection was be contained at this index case. After May 2006 H5N1 HPAIV was found in only one more isolated case involving a juvenile black swan (*Cygnus atratus*) kept in the zoo of the city of Dresden, Saxony.

A re-occurence of the HPAI H5N1 virus was noticed at the end of June 2007 in wild birds at six different locations in the Southeast of Germany (federal states of Bavaria, Saxony and Thuringia). At the same time outbreaks in poultry and a mute swan in the Czech Republic and three mute swans in France were reported. Until to day, 96 wild birds, the majority being mute swans and black-necked grebes (*Podiceps nigricollis*) had succumbed to the infection. In addition, HPAIV H5N1 was also found in a small backyard holding of ducks and geese in Thuringia, where a single goose was found to be infected.

The current study was conducted to elucidate the phylogenetic relationships of the German H5N1 HPAIV isolates to gain further insights into the origin of the virus and to provide clues highlighting putative ways of introduction. Also, analysis of the receptor-binding HA of the feline and the mustelid H5N1 isolate was done to reveal any possible adaptation towards mammals.

Material and Methods

Virus isolation

Viruses were isolated between February and May 2006 and in June and July 2007 from wild birds in Germany and from one mixed poultry holding located in Saxony. Viruses for this study were selected from a panel of 383 H5N1 HPAIV positive wild birds in order to represent geographically different parts of Germany, as many different species as possible

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and the two temporally separated waves of outbreaks. Initially, the original material was characterized by real-time RT PCR assays targeting fragments of the M, N1, and H5 genome segments including the sequence encoding the HA_0 cleavage site (Hoffmann et al., 2007). In addition, a virus isolate from a common teal (*Anas crecca*), sampled in autumn 2005 on the Wadden Sea Island of Foehr, Germany, which was characterised by hemagglutination inhibition (HI), RT-PCR assays and sequencing of the HA_0 cleavage site as an H5N1 virus of low pathogenicity, was included in the study. All investigated H5N1 viruses were isolated and propagated in embryonated eggs as described previously (Anonymus, 2006). All work subject to handling of material containing infectious HPAIV was conducted in bio-safety level 3+ facilities.

RNA isolation, RT-PCR and sequencing

RNA was prepared from allantoic fluid by use of the QIAamp Viral RNA Kit (QIAGEN) according to the manufacturer's instructions. The complete HA open reading frame was reverse transcribed and amplified according to Hoffmann et al. (2001). Primers were used in a concentration of 40 or 100 pmol/µl, respectively. The program profile for amplification of cDNA was 95°, 5'; 35x (95°, 30"; 58°, 40"; 72°, 3-7'); 72°, 7'; 8°, hold. Alternatively, own primers were used to generate overlapping HA fragments (nt 1 – nt 1450; nt 935 – 1779 referring to sequence DQ464354) for direct sequencing (primer sequences are available upon request). For the characterization of a fragment of the gene encoding neuraminidase subtype 1 a primer pair recommended by the WHO (Wright et al., 1995) which spans nucleotides 490 – 1105 was used. The amplicon was generated with the OneStep RT-PCR Kit (QIAGEN GmbH) as recommended by the manufacturer (program profile: 50°, 30'; 94°, 15'; 35x (94°, 45"; 55°, 45"; 72°, 1'); 72°, 7'; 8°, hold). All RT-PCR products were eluted from agarose gels and purified with the QIAquick Gel Extraction Kit (QIAGEN). HA full-length fragments were cloned into the pGEM-T Easy Vector with the help of the pGEM-T Easy Vector System (Promega Corp.). DNA was sequenced using the Prism BigDye Terminator v1.1 Cycle Sequencing Kit on the DNA sequencer “3130 Genetic Analyzer” (both Applied Biosystems). From each HA full-length RT-PCR product, at least 5 plasmids were selected and sequenced with vector-specific primers from both sites. For a full overlap of forward and reverse sequences sequence-specific internal primers were used (Starick et al., 2000). If full length fragments could not be generated, smaller overlapping amplicons were sequenced directly in both directions by using the same primer pairs as for RT-PCR. Sequence data were assembled and edited with GCG, Version 11.1 (Accelrys Inc.). The nucleotide sequences analyzed here cover nt 29–1735 (HA) and nt 506 –1043 (NA), respectively.

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Phylogenetic analyses

Consensus sequences of the analyzed fragments were generated. Full length HA- and partial NA-sequences were aligned with selected sequences of standard strains representing major clades of H5N1 viruses from GenBank (GCG programs; CLUSTALW; HUSAR, DKFZ Heidelberg). Phylogenetic analysis based on the HA and NA alignments was performed by using the PUZZLE software (Strimmer & von Haeseler, 1996). For maximum-likelihood (ML) tree reconstructions, 1000 puzzling steps and the Hasegawa model for nucleotide substitution were used. Dendrograms generated by PUZZLE were visualized by using the TREEVIEW software (Page, 1996). Similarly, alignments were examined by the neighbor-joining (NJ) distance matrix algorithm (MEGA 4.0) using maximum composite likelihood techniques to estimate the transversion/transition bias and nucleotide substitution patterns while assuming rate variation among sites and substitution pattern heterogeneities among lineages. Finally, a maximum parsimony (MP) approach (heuristic search) was used (MEGA 4.0 software; Tamura et al., 2007).

Results

Generation and characterization of HA and NA sequences

A total of 27 HPAIV H5N1 isolates collected during two outbreaks among wild birds in 2006 and 2007 from all affected federal states of Germany were investigated (Table 1). They originated from 16 different wild bird species, from an infected turkey flock as well as from a stray cat and a stone marten. For comparison, an LPAIV H5N1 isolate retrieved from a common teal in autumn 2005 was included in the study. Sequences spanning the complete coding part of the HA segment were created for 23 isolates, and for the remaining 5 HAs the sequences cover 93% of the HA coding region (nt 82–1735). The NA genes of the different isolates were partially sequenced (nt 506 -1043).

All isolates investigated shared features characteristic for recent HPAIV H5N1 isolates of Asian origin. The alignment of the deduced amino acid sequences of the H5 HA genes showed that all isolates have the same multiple basic amino acids at the connecting peptide between HA1 and HA2 (PQGERRRKKR * GLF). Isolate WV632/05 used here as an outgroup representative, represents an exception as it possesses a monobasic cleavage site typical of LPAIV (PQKETR * GLF). The polybasic cleavage site is identical to that found in all clade 2 H5N1 viruses isolated in other Asian, European and African countries (Ducatez et al.,

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2006; Salzberg et al., 2007). Based on the amino acid sequence at the HA_0 cleavage site, these new H5N1 influenza viruses are regarded as being highly pathogenic for chickens (Anonymous, 2005). Six potential N-glycosylation sites were localised in the HA1 part for all isolates. Within the receptor binding sites, no amino acid changes were detected including the mammalian isolates obtained from a cat and a stone marten. In addition, all isolates carried HA 238Q and HA 240G (numbering from the initiating methionine residue) at the receptor binding pocket which indicates preferential binding to alpha-2,3-NeuAcGal receptors (Li et al., 2004; Smith et al., 2006a). Further analysis of the HA amino acid (aa) sequences revealed only a few scattered non-synonymous mutations at different positions including the signal peptide (data not shown). The only conspicuous position is HA 403 where D is present preferentially in isolates originating from the South of the country (with the exceptions of R882/06 and R1240/06 which come from the Northern and Central part, respectively, of Germany), and N predominates in Northern isolates including R751/06 from the South of the country.

Full-length sequences of the NA gene of several isolates from swans, one cat and the stone marten revealed the typical deletion of 20 amino acids in the stalk region (position 49-68; data not shown) which is not present in LPAIV WV632/05 (Li et al., 2004; Harder and Werner, 2006, Weber et al., 2007). Amino acid NA 275 specified H for all HP isolates from 2006 and 2007, thus predicting full susceptibility to oseltamivir (WHO, 2005; Smith et al., 2006b).

Phylogenetic analyses

Phylogenetic characterization of the HA gene by ML, NJ and MP methods revealed that the analysed HPAIV H5N1 from terrestrial and aquatic birds as well as from mammals in Germany cluster with Qinghai-like viruses (Fig. 1). Further analyses of the HA gene showed that three different clusters are distinguishable among the H5N1 HPAIV isolates from Germany. These clusters are similar to those reported by Salzberg et al. (2007) as EMA-1 to 3 which, according to the most recent nomenclature should be referred to as subclades 2.2.1 to 2.2.3 (WHO/FAO/OIE H5N1 Evolution Working Group, 2007). Placement of the German isolates in the different clusters coincides, with few exceptions, with the geographic origin of the isolates from the Northern (Fig. 2, subclade 2.2.2) or Southern (Fig. 2, subclade 2.2.1) part of Germany and with the temporal succession of the outbreaks (2.2.1, 2.2.2: 2006; 2.2.3: 2007). Isolate R882/06, although originating from a common gull (*Larus canus*) in Lower Saxony, Northern Germany, and isolate R1240/96 from a tufted duck (*Aythya fuligula*) in Saxony clustered with the 'Southern group'. Vice versa, one isolate from the South of

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Germany (R751/06) belonged to the 'Northern group' as did the turkey isolate R1077/06, obtained from the single affected poultry holding in Germany. Table 2 summarizes the closest relatives of the German isolates of subclades 2.2.1 to 3, respectively, according to a BLAST search and pairwise comparison to H5N1 HPAIV HA sequences from other European countries (one per country).

This classification into three groups is mirrored by the NA sequences, although only 538 nt were included in the analysis for most isolates (data not shown). Nucleotide positions 743, 783, 1007, 1071 and 1083 (numbering from the start codon) define the clusters 2.2.2 and 2.2.1. Mutations observed at two of these positions (248, 336) were non-synonymous and characteristic for the two groups (2.2.2: 248N, 336D; 2.2.1: 248S, 336G). This differentiation also included the three isolates R751/06, R882/06, and R1240/06 which represented exceptions concerning the geographical restriction also on the basis of HA sequences. In isolates of cluster 2.2.3 248N and 336G were present.

Discussion

The investigations of H5N1 HPAIV isolates originating from Germany in 2006 and 2007 was conducted in order to (i) identify possible sources and routes of introduction and (ii) molecularly characterize potential species-specific differences in the viral glycoprotein genes, particularly between avian and mammalian hosts.

Phylogenetically, three clusters of highly pathogenic H5N1 avian influenza viruses could be distinguished on the basis of their HA and NA gene sequences (2.2.1 to 3). Geographic and temporal restrictions of these clusters were evident as 2.2.2-like viruses were found in the North of Germany while 2.2.1-like isolates were predominantly found in the Southern part of the country (Fig. 2). Differences between these clusters were minor but consistent. The position HA 403 specified aa D for 2.2.2 and N for 2.2.1 and served to differentiate these clusters. In addition, aa positions 247 and 335 of the neuraminidase similarly revealed marker-like properties for clusters 2.2.2 and 2.2.1. All 2.2.3-like isolates originated from the 2007 outbreak in Southeastern Germany. No 2.2.3-like isolates were detected in Germany in 2006.

Recently, Salzberg et al. (2007) analysed 71 complete genomes of H5N1 HPAIVs isolated from Europe, the Middle East, Russia and Africa. Based on their data three different

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phylogenetic clades of H5N1 viruses from Europe-Middle-East-Africa (EMA) were distinguished. Analyses of our data on a larger phylogenetic scale showed that the isolates from the North of Germany fall into the EMA group 2 (subclade 2.2.2), while those from the South cluster with EMA-1 (subclade 2.2.1) and those from 2007 with EMA-3 (subclade 2.2.3; Fig. 1). An H5N1 isolate obtained from a common teal in autumn 2005 in the Northwestern part of Germany, i.e. four months before the detection of H5N1/Asia-like viruses in Germany, proved to be of low pathogenicity and clustered with other LPAIVs of recent Eurasian origin (data not shown). Pairwise comparisons of randomly selected H5N1 HPAIVs isolated in 2006 and 2007 in other European countries with consensus sequences of subclades 2.2.1 to 3 revealed a very close relationships of these viruses to subclades 2.2.1 and 2 , respectively. However, subclade 2.2.3 proved to be more distinct (Table 2). No distinct patterns as to the geographical origin of ancestors of subclades 2.2.1 to 3 viruses were evident. Nevertheless, the striking homogeneity of viruses of subclade 2.2.2 suggests that a single introduction caused the epidemic outbreak on the Baltic island of Ruegen. From this epicentre the infection spread radially towards the West and South. Sequences of subclade 2.2.1, in contrast, showed more diversity. This might be due to the longer persistence of the infection in the South where infections could be detected until early May as compared to late March in the North, and, consequently, the chance for an accumulation of mutations. On the other hand, repeated introductions of closely related GER-B like viruses could also have occurred (Rinder et al., 2007). For subclade 2.2.3 closest publicly available sequences originate from wild birds in Mongolia and from poultry in Afghanistan. There is to our knowledge only a single HPAIV H5N1 virus (A/mute swan/Italy/742/2006) of this EMA-3/2.2.3 subclade which has been detected in 2006 in Europe.

No host-related differences were detectable in the sequences analysed. This includes isolates from 16 different aquatic and terrestrial avian species as well as a cat and a stone marten (see also Weber et al., 2007). The mammalian viruses proved to be similar to those circulating at the same time in wild birds indicating that infection of these mammals occurred most likely by scavenging on wild bird carcasses. In addition, the results show that a single passage of the virus in another species and even in mammals is not necessarily associated with changes in the receptor-binding sites.

Our data suggest the simultaneous introduction in early 2006 of two closely related but distinct H5N1 variants into the wild bird population of Germany. The source of these viruses and the exact time of introduction could not be identified. However, based on the very high degree of similarity between isolates within each of the two respective clusters and the

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identification of closely related H5N1 viruses from southern and central Russia an introduction, possibly via wild birds on winter escape from these regions, early in 2006 appears to be a highly likely scenario. The re-occurrence of HPAIV H5N1 in 2007 appears to be related to a new incursion. These viruses belong to subclade EMA-3 which had not been detected in Germany previously. Its sources and routes of introduction remain to be resolved.

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Legend to figures

Figure 1.

Phylogenetic analysis of the coding part (nt 29-1735) of the HA gene of 24 H5N1 HPAIV isolates obtained from naturally infected wild birds and mammals in spring 2006 in Germany. Phylogenies were established by maximum likelihood (quartet puzzling); neighbor-joining and maximum parsimony analyses resulted in similar topologies. EMA (Europe-Middle East-Africa) clades refer to Salzberg et al. (2007) and have been designated in the text “subclades 2.2.1 (EMA 1) to 2.2.3 (EMA 3)” according to the WHO/FAO/OIE H5N1 Evolution Working Group (2007).

Figure 2.

Detection by real time RT PCR and localization of 439 wild birds infected with HPAIV H5N1 in Germany in spring 2006 (red dots) and in summer 2007 (purple dots). Blue dots represent four mammals found to be H5N1-infected. Phylogenetic analysis of the HA and NA genes of 27 isolates allowed to distinguish, in 2006, a Northern (yellow triangles, subclade 2.2.2) from a Southern cluster (orange squares, subclade 2.2.1) and, in 2007, a third subclade (green stars, subclade 2.2.3) suggesting at least three different introductions of H5N1 into Germany, Outbreaks in poultry are marked with a separate circle around a dot.

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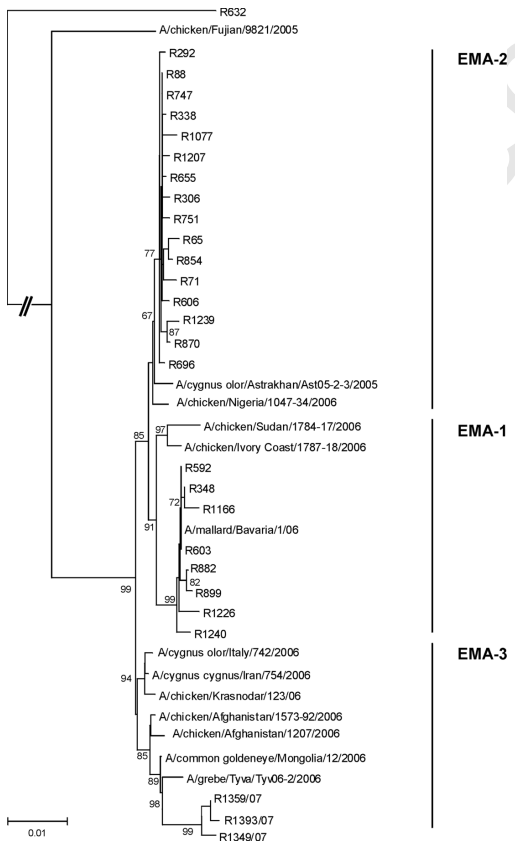

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Figure 2.

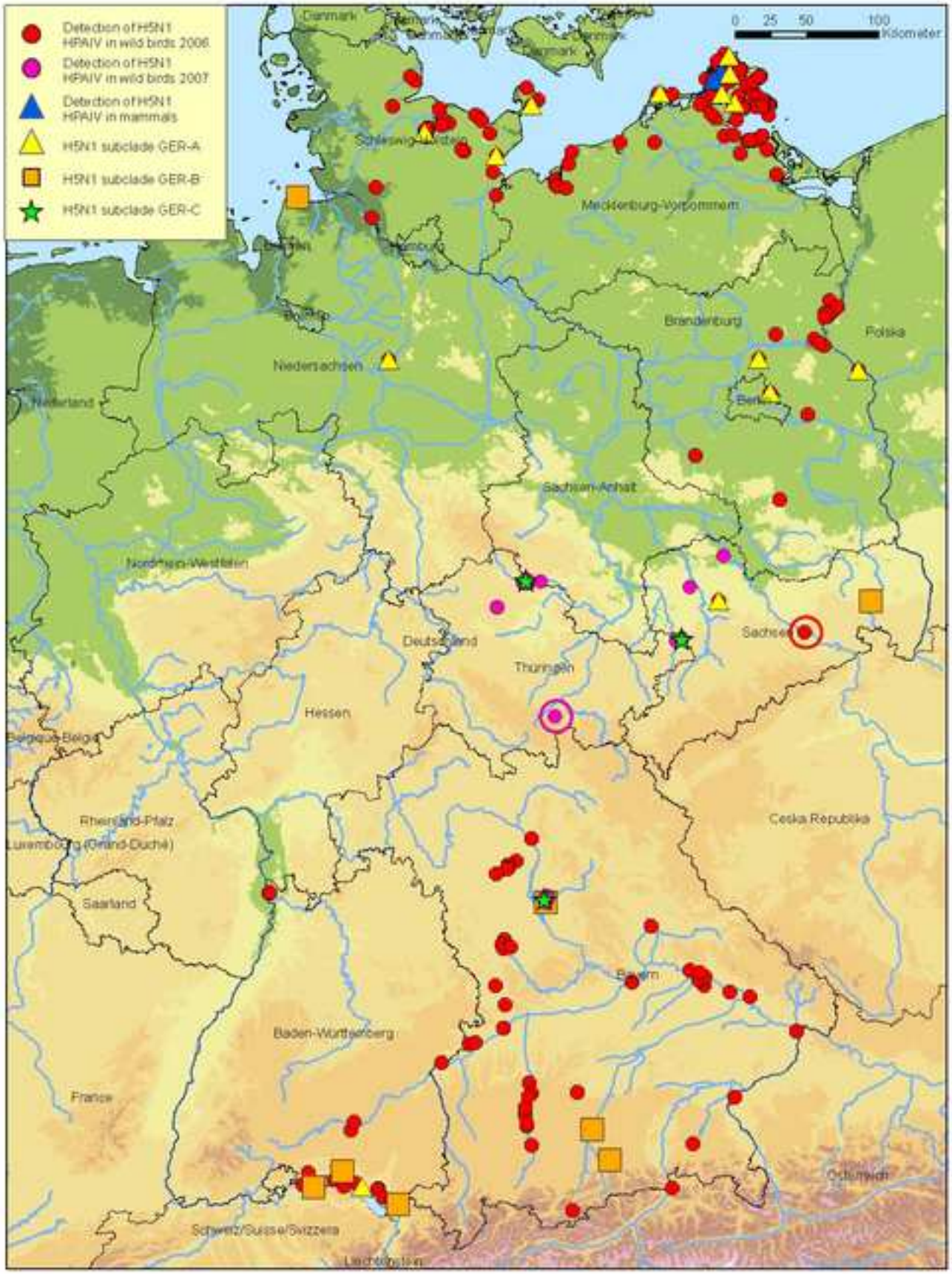

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Table 1. Identification, geographic origin and sequence data of HPAIV H5N1 isolates originating from Germany 2006.

Isolate	Geographic region[1]	HA-full length (nt 29-1756)	HA-partial (nt 82-1735)	NA-partial (nt 526-1063)
Subclade 2.2.1				
A/Aythya ferina/Germany/R348/06	South	AM403465		AM403139
A/Aythya ferina/Germany/R592/06	South	AM403466		AM403140
A/Anas spec./Germany/R603/06	South	AM403467		AM403141
A/Larus canus/Germany/R882/06	North	AM408215		AM403148
A/Falco tinnunculus/Germany/R899/06	South		AM408211	AM403149
A/Bubo bubo/Germany/R1166/06	South	AM403473		AM403151
A/Podiceps cristatus/Germany/R1226/06	South		AM408212	AM403153
A/Aythya fuligula/Germany/R1240/06	Central	AM408216		AM403155
Subclade 2.2.2				
A/Cygnus cygnus/Germany/R65/06	North	AM403460		AM403133
A/Branta canadensis/Germany/R71/06	North	AM403461		AM403134
A/Cygnus cygnus/Germany/R88/06	North	AM403462		AM403135
A/Phalacrocorax carbo/Germany/R292/06	North		AM408209	AM403136
A/Larus argentatus/Germany/R306/06	North	AM403463		AM403137
A/Anas platyrhynchos/Germany/R338/06	North	AM403464		AM403138
A/Felis catus/Germany/R606/06	North	AM403468		AM403142
A/Anser anser/Germany/R696/06	North		AM408210	AM403144
A/Fuliva atra/Germany/R655/06	North	AM403469		AM403143
A/Martes foina/Germany/R747/06	North	AM492165		AM492166
A/Aythya fuligula/Germany/R751/06	South	AM403470		AM403145
A/Cygnus olor/Germany/R854/06	North	AM403471		AM403146
A/Buteo buteo/Germany/R870/06	North		AM408213	AM403147
A/Meleagris gallopavo/Germany/R1077/06	Central	AM403472		AM403150
A/Branta canadensis/Germany/R1207/06	North	AM403474		AM403152
A/Ciconia ciconia/Germany/R1239/06	North	AM403475		AM403154
Subclade 2.2.3				
A/Cygnus olor/Germany/R1349/07	South	AM749442		AM749445 *
A/Cygnus olor/Germany/R1359/07	Central	AM749443		AM494444
A/Podiceps nigricollis/Germany/R1393/07	Central	AM773724		AM773725
Outgroup				
A/Anas crecca/Germany/WV632/05 (H5N1-*LPAI*)	North	AM408214		AM408217

* complete sequence

[1] Geographic area of Germany

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Table 2. Pairwise comparison of GER-A and –B consensus and one Ger-C representative nucleotide sequences to HA full-length coding sequences from Europe; one per country (GCG, Version 11.1; Accelrys Inc.)

		Percentage identity		
Accession no	**Strain designation**	**2.2.2 consensus**	**2.2.1 consensus**	**2.2.3 consensus**
DQ434889	A/Cygnus olor/Astrakhan/Ast05-210/2005	99.60	98.97	98.30
DQ407519	A/Meleagris gallopavo/Turkey/1/2005	98.99	98.82	98.07
CY016819	A/Cygnus olor/Croatia/1/2005	99.83	99.19	98.54
CY017043	A/Cygnus olor /Slovenia/760/2006	99.31	99.13	98.42
CY016795	A/Anas platyrhynchos/Italy/835/2006	99.13	99.08	98.07
DQ458992	A/Anas platyrhynchos/Bavaria/1/2006	99.24	100	98.18
EF110519	A/Fulica atra/Switzerland/V544/06	99.06	99.82	98.01
EF395845	A/Cygnus olor /Austria/216/2006	99.37	99.2	98.36
DQ515984	A/Cygnus olor/Czech Republic/5170/2006	99.14	98.99	98.07
EF474450	A/Gallus gallus/Moscow/2/2007	98.75	98.4	98.18
EF446771	A/Anser domesticus/Hungary/2823-2/2007	99.48	99.31	98.42
EF441263	A/Meleagris gallopavo/England/259/2007	99.41	99.35	98.40
AM403462	A/Cygnus cygnus/Germany/R88/06	100	99.06	98.48
AM408216	A/Aythya fuligula/Germany/R1240/06		100	98.18
AM773724	A/Podiceps nigricollis /Germany/R1393/07			100

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