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Molecular and phylogenetic analysis of the H5N1 avian influenza virus caused the first highly pathogenic avian influenza outbreak in poultry in the Czech Republic in 2007

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Abstract

On 19th July 2007 re-occurrence of the H5N1 highly pathogenic avian influenza (HPAI) virus was noticed in Europe. The index strain of this novel H5N1 lineage was identified in the Czech Republic where it caused historically the first HPAI outbreak in commercial poultry. In the present study we performed molecular and phylogenetic analysis of the index strain of the re-emerging H5N1 virus lineage along with the Czech and the Slovak H5N1 strains collected in 2006 and established the evolutionary relationships to additional viruses circulated in Europe in 2005-2006. Our analysis revealed that the Czech and the Slovak H5N1 viruses collected during 2006 were separated into two sub-clades 2.2.1 and 2.2.2 which predominated in Europe during 2005-2006. On the contrary the newly emerged H5N1 viruses belonged into a clearly distinguishable sub-clade 2.2.3. Within the sub-clade 2.2.3 the Czech H5N1 strains showed the closest relationships to the simultaneously circulated viruses from Germany, Romania and Russia (Krasnodar) in 2007 and were further clustered with the viruses from Afghanistan and Mongolia circulated in 2006. The origin of the Czech 2007 H5N1 HPAI strains was also discussed.

1. Introduction

The highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype, commonly called “bird flu”, is a serious pathogen causing severe disease outbreaks in poultry. Since 1997, the first lethal human infection (Subbarao et al., 1998), there has been an increasing number of human deaths which puts the H5N1 virus high as a causative agent of the next human influenza pandemic. Recently, however, the infection in humans has been exhibiting sporadic character and no human to human transmission of the virus was consistently observed.

The H5N1 HPAI virus was firstly detected in China in 1996 (Xu et al., 1999). From 1996 to 2006 the virus underwent multiple genotype alterations through reassortment with other co-circulating avian influenza viruses (AIV) or pre-existing H5N1 genotypes (Guan et al. 1999; Hoffmann et al. 2000; Guan et al., 2002; Chen et al., 2004; Li et al., 2004; Chen et al., 2006a) and evolved along nine major H5 hemagglutinin lineages (H5N1 Evolution Working Group WHO/FAO/OIE, 2007). An unresolved selection mechanism led to the generation of a Z genotype which has been predominating since 2002 (Li et al., 2004; Chen et al., 2006a).

In May 2005 an H5N1 virus caused massive HPAI outbreak in waterfowl at Qinghai Lake in western China (Chen et al. 2005, Liu et al. 2005). During 2005-2006 the Qinghai-like viruses spread through Eurasia and Africa and caused the pan Eurasian-African H5N1 HPAI outbreak which represents the largest outbreak wave during the recorded history of the H5N1 virus. Based on the H5N1 Evolution Working Group classification system the Qinghai-like strains belong to the H5N1 virus lineage 2 where they represent a separate clade designated as 2.2. Within the Qinghai-lineage the European, Middle East and African viruses can be separated into three sub-clades 2.2.1, 2.2.2 and 2.2.3 (H5N1 Evolution Working Group WHO/FAO/OIE, 2007) previously designated as EMA 1, EMA 2 and EMA 3 (European, Middle East and African; Salzberg et al., 2007).

The first detection of the H5N1 HPAI in Europe can be dated from October 2005 when outbreaks were reported within a short time period from Turkey (6th October 2005), Romania (7th October 2005) and Croatia (20th October 2005) (WHO, OIE). During following few months the infection overran almost the whole continent and caused numerous outbreaks in wild birds and poultry (WHO, OIE, Bragstad et al. 2007; Nagy et al., 2007; Weber et al, 2007; Starick et al., 2007). From summer of 2006 there was a gradual

decline in the frequency of the H5N1 outbreak reports throughout Europe and the virus has apparently disappeared in the fall of 2006 with no records on the OIE official web pages.

On 19th January 2007, an H5N1 virus caused outbreaks in geese flocks in Hungary and subsequently, on 27th January 2007 in the United Kingdom (UK) (WHO, OIE). However, during the following five months no further H5N1 outbreaks were reported in Europe till 19th June 2007 when the virus re-emerged in the Czech Republic. The infection was detected in two Czech regions, Pardubice and South Moravia. The outbreak in Pardubice region was initially noticed in a turkey farm and represents the first HPAI outbreak ever detected in commercial poultry in the Czech Republic. In South Moravia one mute swan (*Cygnus olor*) was found infected.

In the present study we performed molecular and phylogenetic analyses of the representative H5N1 strains, which caused HPAI outbreaks in the Czech Republic in 2007. We focused on: i) molecular-genetic end phylogenetic characterization of the H5N1 virus strains which circulated in the Czech Republic and the Slovak Republic in 2006, ii) characterization of the H5N1 strains detected in the Czech Republic during the HPAI outbreak in 2007, iii) determination of the evolutionary relationships among the Czech, Slovak and additional European H5N1 strains which circulated during 2006 and 2007, iv) discuss the origin of the H5N1 strains collected in the Czech Republic in 2007.

2. Materials and methods

2.1. Virus detection and identification

Pooled organ suspensions (brain, trachea, lungs, liver and intestines) prepared in PBS buffer were used for the diagnosis of the H5N1 AIV (avian influenza virus) during the outbreak in a turkey farm in Tisova and from a mute swan in South Moravia region. From secondary three outbreaks in Pardubice region (see the results) cloacal and tracheal swabs were analysed. The RNA was extracted via MagNAPure Compact and/or MagNAPure LC robotic workstations (Roche) and a TaqMan probe based real-time RT-PCR assay (OneTube RT-PCR kit, Qiagen) designed for the detection of the gene segment encoding the matrix protein (Diagnostic manual for avian influenza, 2006;) were employed as the first screening line for positive/negative sample discrimination. Reactions were performed on LightCycler 1.2 (Roche) and MJ MiniOpticon (Bio-Rad) platforms. The H5 hemagglutinin (HA) subtype was determined via real-time RT-

PCR assay (Diagnostic manual for avian influenza, 2006) and the N1 neuraminidase (NA) was subtyped with a TaqMan real-time RT-PCR assay using the primer pairs selected from the conserved region of the avian N1 NA gene segment (5'→3'): forward primer SVUP-N1-F AGY GGN TTT GAR ATG ATT TGG GA, reverse primer SVUP-N1-R GAC TTG TCA ATG GTG AAT GGC A and the probe SVUP-N1-X FAM-TGG TCT TGG CCA GAC GGT G-MGB (Nagy et al., unpublished). The reaction (OneTube RT-PCR kit, Qiagen) was performed in a final volume of 25µl and the concentration of MgCl₂ was adjusted to 4mM. The thermocycling profile (LightCycler 1.2) was the following: 30 min at 50°C and 15 min at 95°C and then 45 cycles of 10sec at 95°C, 20sec at 60°C and 10sec at 72°C, with the 60°C to 72°C ramp rate of 0.5°C/sec. The signal acquisition was measured at the end of the 60°C period. For molecular pathotyping of the H5N1 AIV viruses the amplicon enclosing the cleavage site region of the H5 HA generated by the Kha1-Kha3 primer pair (Diagnostic manual for avian influenza, 2006) was sequenced on the Applied Biosystems 3130 genetic analyser.

2.2 Phylogenetic analysis

In an effort to characterise the H5N1 AIV viruses detected in the Czech Republic in 2006-2007 and determine their evolutionary relationships to the European H5N1 strains circulated during 2005-2007 all of the genome segments of the index strain A/turkey/Czech Republic/10309-3/07 and further A/chicken/Czech Republic/11242-38/07 and A/Cygnus olor/Czech Republic/10732/07 (all abbreviated as CZE/07 strains) were sequenced except the termini used as a primer binding regions (Table 1). This virus set was further supplemented with the strains circulated in the Czech Republic (Table 1, strains 4-6 and 9-16; further abbreviated as CZE/06 strains) and the Slovak Republic (Table 1, strains 7-8; further abbreviated as SVK/06 strains) in 2006. The genome segments encoding the polymerase complex were amplified and sequenced with the primers described by Li et al. (2007) and the segments for MP and NS proteins according to Hoffmann et al. (2001). The segments encoding the HA, NP and NA genes were amplified and sequenced via primer set designed in our laboratory (available on request). All genome segments of the CZE/06, SVK/06 and the CZE/07 strains were further compared with the representative H5N1 sequence data deposited in the Influenza Virus Resource public database (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>) (Bao et al., 2007). Sequences were aligned with

ClustalX (Jeanmougin et al., 1998) and the alignments were edited by BIOEDIT 7 (Hall, 1999). Neighbour-joining (NJ), maximum parsimony (MP) and maximum-likelihood (ML) trees were calculated for each genome segment using PHYLIP 3.63 (J. Felsenstein, <http://evolution.genetics.washington.edu/phylip.html>). For the NJ Kimura's two-parameter nucleotide substitution model was implemented. The robustness of nodes was evaluated by performing 1000 (for NJ and MP) and 100 (for ML) bootstrap replicates. Trees were edited by the Tree Explorer tool in Mega 4 (Tamura et al., 2007). The sequences were deposited in GenBank under the following accession numbers: EU443533-EU443604.

3. Results

3.1 Course of the AIV outbreaks in the Czech Republic in 2006 and 2007.

The first H5N1 HPAI virus occurrence in the Czech Republic is dated from 23rd March to 8th April 2006 when a total of twelve mute swans were found dead and positive for the H5N1 virus in different areas of the South Bohemia region (Nagy et al., 2007; Figure 1). Subsequently on 12th May 2006 two additional dead mute swan positive for the H5N1 AIV were collected in around 200km distant Kostice, South Moravia region. In spite of ongoing AIV surveillance no further H5N1 outbreaks have been identified since the end of May 2006.

On 19th June 2007 an H5N1 HPAI outbreak was noticed on a turkey farm in the Czech Republic. The farm was located in Tisova, Pardubice region and held around 6000 birds for meat production. Shortly after, on 25th June 2007 the virus was detected in one dead mute swan found in approximately 140 km distant Lednice, south Moravia region.

The H5N1 virus infection in Pardubice region spread into three chicken farms, on 27th June 2007 in Norin and on 11th July 2007 in Chocen and Natreby, situated in a 3-5 km distance from the place of the index outbreak in Tisova.

3.2 Molecular-genetic characterization of the CZE/06, SVK/06 and the CZE/07 viruses

All of the H5N1 viruses characterised in this study had the PQGERRRKKR/G cleavage site motif which classified the CZE/06, SVA/06 and the CZE/07 strains among HPAI viruses. The remaining molecular features like the receptor binding preference, 20 amino acid-long deletion in the N1

neuraminidase, five amino acid deletion and Alanin at position 149 in the NS1 protein (Matrosovich et al., 1999; Stevens et al., 2006; Li et al., 2006) as well as to the critical positions of the NA and M2 proteins predicting oseltamivir and amantadin sensitivity (Gubareva et al., 2001; Suzuki et al. 2003) were identical to the previously characterised H5N1 viruses (reported elsewhere). These molecular markers were also identical in all European viruses analysed in our study (data not shown).

The H5 amino acid sequence predicts seven possible N-linked glycosylation sequons, six of which (HA1 21, 33, 169, 197, 289 and HA2 154, H3 numbering) were found as highly conserved among the European 2005-2006 H5N1 strains (data not shown). The glycosylation site at position 158 located at the distal globule of the H5 HA molecule, capable to mask the antigenic sites (Stevens et al., 2006) and alter the receptor binding profile (Iwatsuki-Horimoto et al., 2004) was not present in the CZE/06, SVA/06 and the CZE/07 strains.

Hatta and colleagues (2001) found that substitution at position 627 (E/K) of the PB2 protein is responsible for alteration of the H5N1 virus virulence in mice. The CZE/06, SVA/06 and the CZE/07 strains also showed E, K diversity at position 627. While the CZE/06 viruses collected in South Bohemia (strains 5170, 6111, Table 1) and the SVK/06 viruses exhibited Glutamic acid the CZE/06 strain from South Moravia (strain 10814) and the CZE/07 viruses possessed Lysine.

In effort to reveal possible sequence variations between the H5N1 virus strains collected from different farms within the same outbreak area the entire genome of A/turkey/Czech Republic/10309-3/07 (Tisova) and A/chicken/Czech Republic/11242-38/07 (Chocen) was compared. Nucleotide sequence alignment indicated that the turkey and chicken strains shared 100% sequence similarity for the first 7 genome segments. The NS gene segment possessed a single nucleotide difference at position 566 (C/T) which did not result in amino acid change.

3.3 Phylogenetic analysis of the CZE/06, SVK/06 and the CZE/07 viruses

Phylogenetic tree calculated separately for each genome segment showed that the entire genome of the CZE/06, SVK/06 and the CZE/07 strains was “Qinghai-like” i.e. the viruses re-emerged without genetic reassortment (data not shown).

The H5 gene tree constructed on the basis of the available European sequences from 2005-2007 indicated separation into three sub-clades 2.2.1, 2.2.2 and 2.2.3 (Figure 2). Sub-clades 2.2.1 and 2.2.2 contain predominantly the viruses circulated in 2005-2006 while the viruses circulated since June 2007 were included in the sub-clade 2.2.3. Phylogenetic analysis further revealed that the Czech strains belonged into all three H5 sub-clades.

The CZE/06 viruses from South Bohemia and the SVK/06 strains were clustered into sub-clade 2.2.1 which showed the greatest divergence among the sub-clades. Within the sub-clade 2.2.1 the European H5N1 strains can be further divided into three branches (well supported with bootstrap values) which, following the established H5 gene tree nomenclature, were designated as 2.2.1.1-3. The first branch 2.2.1.1 (bootstrap value 93.3) included the SVK/06 strains, the CZE/06 viruses from South Bohemia and further comprised with European H5 strains collected in 2006 from Germany, Italy, France, Austria and Slovenia. Interestingly, one of the first European H5N1 strain, A/turkey/Turkey/1/05, belonged also to this branch. The second branch of the sub-clade 2.2.1 (bootstrap value 99.5) contained mainly German, French and Swiss 2006 viruses and the third branch 2.2.1.3 (bootstrap value 95.4) clustered the Hungarian and the United Kingdom viruses from January 2007.

In comparison to the South Bohemian CZE/06 and the SVK/06 strains the CZE/06 viruses from South Moravia were separated into the sub-clade 2.2.2. This obviously less heterogeneous sub-clade is further comprised by viruses mainly from Germany and Denmark. In addition the Scottish H5N1 strain from 2006 and the Croatian strain from 2005 were also included in this sub-clade.

Finally, the CZE/07 strains showed different clustering pattern both from the CZE/06 and the SVK/06 strains and generally also from the European H5N1 viruses collected in 2005-2006 and formed a separate sub-clade 2.2.3 with the closest relationships to the strains from Romania, Germany and Russia (Krasnodar) which circulated from summer 2007. Interestingly one of the European H5N1 viruses from 2006 namely A/Cygnus olor/Italy/742/06 belonged also into the sub-clade 2.2.3. However, this Italian strain formed a separate branch from the CZE/07-like sequences with the closest relationships with the viruses from Russia, Iran and Dagestan collected in 2006.

4. Discussion

The pan Eurasian-African H5N1 HPAI outbreak during 2005-2006 showed for the first time that this potentially pandemic virus can be transmitted into Europe. Although since late 2006 the infection has apparently disappeared from Europe (OIE) the virus remained in circulation in the Middle East, Russia and South Asia providing potential sources for the virus re-introduction into Europe.

Considering the Czech H5N1 HPAI outbreaks from the geographical point of view, three regions of the Czech Republic were affected: South Bohemia in 2006, Pardubice in 2007 and South Moravia either in 2006 and 2007. The H5N1 virus in South Moravia in 2006 was detected in Kostice (strains A/Cygnus olor/ Czech Republic/10662/06 and A/Cygnus olor/ Czech Republic/10814/06) while in 2007 in Lednice (strain A/Cygnus olor/Czech Republic/10732/07). The very close distance between these two areas (approximately 15 km) along with the consequential appearance of the H5N1 virus in this region highlights the importance of South Moravia as one of the critical regions for the future AIV surveillance approaches in the Czech Republic.

Phylogenetic analysis of the CZE/06 viruses from South Bohemia (March 2006, Nagy et al., 2007) and South Moravia (May 2006) revealed separation into two distinct sub-clades 2.2.1 and 2.2.2. The relatively close geographic distance between these two outbreaks localities (the average distance between Ceske Budejovice and Kostice is around 200km) indicated co-circulation of two H5 sub-clades within a relatively small geographic area. Similar results in terms of co-circulation of two H5 sub-clades were found by Starick (and colleagues, 2007) after the phylogenetic analysis of the German strains from 2006. On the other hand the representative Swiss, French and Danish strains collected in 2006 showed relative uniformity and belonged into single sub-clade 2.2.1 or 2.2.2.

Evolution of the European 2005-2006 H5 HA strains along two sub-clades suggests two main introductions of the H5N1 virus into Europe. However, the marked genetic diversity especially within the sub-clade 2.2.1, with in at least three recognizable branches, suggests multiple imports of closely related strains. Similar conclusion was drawn after the phylogenetic analysis of the German H5N1 strains (Rinder et al., 2007, Starick et al., 2007).

The CZE/07 strains detected since June 2007 were clearly separated from the CZE/06, SVK/06 as well as other European 2005-2006 strains into the related sub-clade 2.2.3. The presence of the German

(Starick et al., 2007), Romanian and Russian 2007 H5N1 viruses within this sub-clade indicates common origin with the CZE/07 strains. However, the H5N1 strain collected in Italy during 2006 (A/Cygnus olor/Italy/742/06) and situated within the sub-clade 2.2.3 indicates that this H5 lineage is evidently not entirely novel in Europe. The outbreak wave started with the appearance of the CZE/07 viruses, however, represents a stable emergence of the sub-clade 2.2.3 in Europe.

Interestingly, the close relationships of the CZE/07 strains to the viruses detected in Russia in 2006-2007 and in Mongolia and Afghanistan in 2006 than to the 2005-2006 European strains traced the origin of the CZE/07 viruses in Asia. Based on this we suppose that the European H5N1 viruses emerged in June 2007 resulted from a novel re-introduction from Middle East or Asia. Moreover, closer relationships of the CZE/07 strains to the Asian 2006 isolates (Russia: Tuva, Tyva; Mongolia and Afghanistan) than to the A/Cygnus olor/Italy/742/06 strain (bootstrap support 90%) also supports the re-introduction scenario of the CZE/07-like H5N1 viruses into Europe.

The CZE/06, CZE/07 and the SVK/06 H5N1 strains similarly like the European viruses analysed in this study share the common molecular features determining the receptor binding properties, pathogenicity, glycosylation pattern and amantadin and oseltamivir sensitivity. Variation was seen only at the position PB2 627, which can be traced to the outbreak at Qinghai Lake (Chen et al., 2006b). The H5 amino acid sequence predicts seven conserved N-linked glycosylation sequins. The position 197 of H5 molecule carries an unusual glycosylation sequon NPT where the Prolin residue is situated in the vicinity of the Asparagin. Gavel & Heijne (1990) previously found that Proline within the glycosylation consensus site strongly reduces the likelihood of N-glycosylation. In addition, no carbohydrate attachment was observed at the position 197 of the solved H5 HA crystal structure of A/Vietnam//1203/2004 (Stevens et al. 2006). Therefore, it is highly probable that the position 197 of the H5N1 viruses is not glycosylated. However this presumption needs experimental proof.

The question of how was the virus transmitted into Europe, which carriers and transmission routes were involved, remains to be answered. The sudden appearance of the H5N1 HPAI virus in central Europe, from the geographical point of view, is without any apparent epizootologic conjunctions. From February to June 2007, no H5N1 HPAI virus activity was reported throughout Europe (OIE, 2007) and the appearance of the virus in the Czech Republic in June 2007 did not correspond to any significant wild bird migration

activity in the region (P. Bergmann, Czech Society for Ornithology, personal communication). Except one mute swan found dead in South Moravia region no additional H5N1 virus was detected in wild birds in the Czech Republic in spite of ongoing AIV surveillance. The outbreak in Germany was, however, followed with virus detection in numerous mute swans or black-necked grebes (*Podiceps nigricollis*) (Starick et al., 2007). Similarly, the H5N1 virus during the outbreak in France was detected mainly in swans (OIE). Therefore we consider the wild birds as the most probable way of virus re-introduction into the Czech Republic. Rather it seems that the AIV surveillance in the Czech Republic did not possess sufficient sensitivity to detect the H5N1 virus in the wild bird reservoir before its transmission into commercial poultry farms.

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Fig. 1. Map of the H5N1 HPAI outbreaks in the Czech Republic and the Slovak Republic “Czechoslovakia” in 2006 and 2007. The SVK/06 viruses are designated in green, the CZE/06 viruses from South Bohemia are in blue and the CZE/06 H5N1 strains sampled in South Moravia (Kostice) are in purple. The CZE/07 viruses from Pardubice and South Moravia (Lednice) were designated as a red square and a red dot respectively. The colour scheme is corresponding with Fig.2. Blue square: strains 4, 5, 9-12 and 14 (the numbering is corresponding with Table 1.) from Ceske Budejovice and Hluboka nad Vltavou; blue dot, strain 13 sampled in Mirochov and blue triangle, strain 15 collected in Orlik. Purple square, strains 6 and 16 from Kostice; green square, strain 8 sampled in Gabčíkovo; green dot, strain 7 from Podunajské Biskupice. Red dot, strain 2 isolated from a swan in Lednice; red square, strains 1 and 3 representing the poultry outbreaks in Tisova and Chocen during 2007.

Fig. 2. Phylogenetic tree of the H5 HA gene of the CZE/06, SVK/06 and the CZE/07 H5N1 strains. The H5 represents an abridged version of a larger tree involved all of the European H5 sequences available during the manuscript preparation (not shown) and retained all of the significant branches seen in the original H5 tree. Trees were generated with maximum-parsimony method in the PHYLIP program (NJ and ML approaches revealed the same relationships) on the basis of nucleotides 82-1680 (1599). Trees were rooted to A/goose/Guangdong/1/96 and the bootstrap values (1000 resamplings) in per cent were indicated at key nodes. Blue, the CZE/06 strains collected in South Bohemia; purple the CZE/06 viruses from South Moravia; green, the SVK/06 H5N1 strains; red, the CZE/07 viruses collected in Pardubice and South Moravia provinces. The colouring is corresponding with Fig. 1.

Figure 1

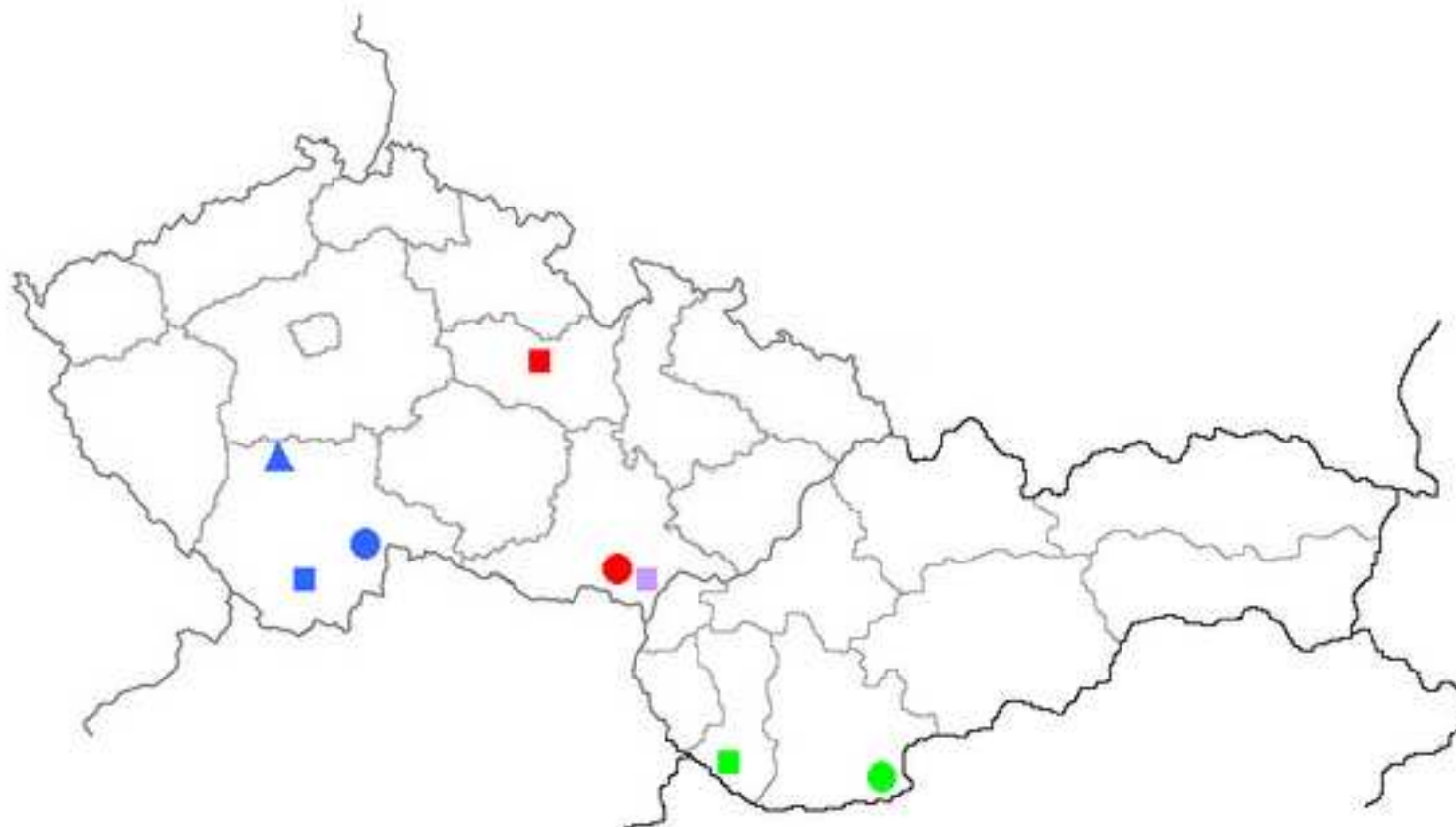


Table 1. The H5N1 viruses analysed in this study

No.	Virus	Sequence analysis	Date and locality of sampling	Region	Specimen
1	A/turkey/Czech Republic/10309-3/07	whole genome	19 th June 2007 Tisova	Pardubice	organ suspension
2	A/cygnus olor/Czech Republic/10732/07	whole genome	25 th June 2007 Lednice	South Moravia	organ suspension
3	A/chicken/Czech Republic/11242-38/07	whole genome	11 th July 2007 Chocen	Pardubice	cloacal swab
4	A/cygnus olor/Czech Republic/5170/06	whole genome	20 th March 2006 Hluboka nad Vltavou	South Bohemia	organ suspension
5	A/cygnus olor/Czech Republic/6111/06	whole genome	29 th March 2006 Ceske Budejovice	South Bohemia	alantoic fluid
6	A/cygnus olor/Czech Republic/10814/06	whole genome	12 th May 2006 Kostice	South Bohemia	alantoic fluid
7	A/mergus albellus/Slovakia/Vh212/06	whole genome	17 th Feb 2006 Podunajske Biskupice	Western Slovakia	alantoic fluid
8	A/peregrine falcon/Slovakia/Vh246/06	whole genome	18 th Feb 2006 Gabcikovo	Western Slovakia	alantoic fluid
9	A/cygnus olor/Czech Republic/5583/06	H5 HA	25 th March 2006 Ceske Budejovice	South Bohemia	organ suspension
10	A/cygnus olor/Czech Republic/5761/06	H5 HA	27 th March 2006 Hluboka nad Vltavou	South Bohemia	organ suspension
11	A/cygnus olor/Czech Republic/5963/06	H5 HA	29 th March 2006 Ceske Budejovice	South Bohemia	organ suspension
12	A/cygnus olor/Czech Republic/6206/06	H5 HA	1 st April 2006 Ceske Budejovice	South Bohemia	organ suspension
13	A/cygnus olor/Czech Republic/6461/06	H5 HA	2 nd April 2006 Mirochov	South Bohemia	organ suspension
14	A/cygnus olor/Czech Republic/7185/06	H5 HA	8 th April 2006 Ceske Budejovice	South Bohemia	alantoic fluid
15	A/cygnus olor/Czech Republic/7208/06	H5 HA	7 th April 2006 Kovarov	South Bohemia	alantoic fluid
16	A/cygnus olor/Czech Republic/10662/06	H5 HA, N1 NA	12 th May 2006 Kostice	South Moravia	organ suspension