New Clade 2.3.4.4b H5N1 Highly Pathogenic Avian Influenza Genotype Detected in Europe in 2021

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Research Article

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Abstract

Despite their widespread distribution, the clade 2.3.4.4b H5N1 viruses have so far only been known in a single genotype variant in Europe. In the study presented, we report the first detection of a new highly pathogenic avian influenza H5N1 genotype in geese and ducks from a backyard farm in the Czech Republic. Phylogenetic analysis has revealed that the Czech H5N1 virus retained the A/Eurasian_Wigeon/Netherlands/1/2020-like backbone with an altered PB2 segment from co-circulating low pathogenic avian influenza viruses.

Main Text

Despite their widespread distribution, the clade 2.3.4.4b H5N1 viruses have so far only been known in a single genotype variant in Europe. In the study presented, we report the first detection of a new highly pathogenic avian influenza H5N1 genotype in geese and ducks from a backyard farm in the Czech Republic. Phylogenetic analysis has revealed that the Czech H5N1 virus retained the A/Eurasian_Wigeon/Netherlands/1/2020-like backbone with an altered PB2 segment from co-circulating low pathogenic avian influenza viruses.

Recurrent outbreaks of H5Nx highly pathogenic avian influenza (HPAI) viruses of clade 2.3.4.4b of the A/goose/Guangdong/1996 (Gs/GD) lineage are of serious concern to the poultry industry worldwide. The evolutional trajectory of the entire clade is characterized by an extremely efficient global spread combined with a high susceptibility for genetic reassortment [1]. Given the zoonotic potential, thorough characterisation of the molecular features and genotypes of emerging 2.3.4.4b H5Nx viruses is of paramount importance to human and animal health. The 2.3.4.4b H5Nx viruses were also responsible for the two largest HPAI outbreaks ever recorded in Europe, in 2016/2017 and 2020/2021[2, 3]. In Europe, five subtypes H5N1, H5N3, H5N4, H5N5, and H5N8 and 19 distinct genotypes were identified during the last HPAI outbreak season (August 2020-September 2021) [3]. While the H5N8 and H5N5 subtypes occurred in different genotypes the H5N1, H5N3 and H5N4 subtypes have so far been identified in only one genomic constellation [3, 4]. In the study presented, we report a new clade 2.3.4.4b H5N1 HPAI genotype.

On 27 September 2021, the National Reference Laboratory (NRL) for avian influenza (AI) identified an outbreak of HAPI of the H5N1 subtype in the Czech Republic. A breeder from the Central Bohemian Region reported to the local veterinary administration the death of five geese from his backyard flock of 32 birds (16 hens and 11 ducks). Clinical signs typical of an AI were observed in one of the ducks. Veterinary inspectors immediately launched an investigation on the farm, took precautionary measures and sent the dead birds to NRL for investigation. HPAI H5N1 has not been detected in the Czech Republic since 2007 [5]. Moreover, the case presented represents a new outbreak of HPAI five months after the H5N8 wave had receded from the country.

Total nucleic acid was extracted from 200µl of pooled organ suspension (MagNAPure Compact, Total NA extraction kit, Roche) and eluted to 50µl. Generic influenza A virus, H5 and N1 subtype specific RT-qPCR

methods and cleavage site sequencing [6-9] revealed the presence of H5N1 HPAI. Subsequently, the remaining poultry on the farm was culled and a three-kilometre protection zone and a ten-kilometre surveillance zone were established by the State Veterinary Administration of the Czech Republic. Within these zones, emergency veterinary measures were declared.

Real-time next-generation sequencing was performed using the nanopore technology (MinION, R9.1 flow cells; Oxford Nanopore Technologies). Briefly, the H5N1 genome was amplified (OneStep RT-PCR Kit, Qiagen) in a final reaction volume of 12.5µl (10µl RT-PCR mix+2.5µl total NA extract; primers available on request). The sequencing libraries were purified (SPRIselect beads; Beckman Coulter) and quantified (QIAxpert; Qiagen). End-preparation, native barcoding and sequencing adapter ligation were performed according to the manufacturer's instructions.

Basecalling was performed by Guppy, and demultiplexing and reference mapping using the RAMPART (Read Assignment, Mapping, and Phylogenetic Analysis in Real Time) module of the ARTIC bioinformatic pipeline set to the concatenated H5N1 genome as a reference. Confirmatory sequencing of the PB2 segment was performed using Sanger sequencing. The consensus genomic segments of the two Czech H5N1/2021 (CZE/18520/2021) strains were submitted to the GISIAD Epiflu database (Acc. No. EPI1921673-88). Phylogenetic analysis was performed using the MEGA10 program [10].

The CZE/18520/2021 H5N1 strains had a multibasic amino acid motif PLREKRRKR*GLF at the cleavage site of the H5 hemagglutinin (HA) gene identical to the 2.3.4.4b strains [1], suggesting a highly pathogenic phenotype in chickens. Blast analysis using the GISAID EpiFlu database indicated that all segments except PB2 of the CZE/18520/2021 H5N1 genome showed high nucleotide sequence identity (≥99.5%) with recent H5N1 strains from the Netherlands and England (Table 1). On the other hand, searching the PB2 segment showed the best match (97.7%) to Eurasian low pathogenic (LPAI) strains of different subtypes.

Phylogenetic analysis of the H5 HA sequences revealed clear partitioning of the CZE/18520/2021 H5N1 within the 2.3.4.4b clade and separation from previously circulated 2.3.2.1c and Eurasian H5N1 viruses [11-13] (Fig 1). In addition, all H5N1 subtypes belonged in a single sub-clade, with A/Eurasian_Wigeon/Netherlands/1/2020 (NL/1/2020) as the prototype, suggesting a monophyletic origin of the H5 hemagglutinin. Similarly, dendrograms constructed for six other genomic segments, i.e., PB1, PA, NP, N1, MP and NS, revealed the closest relationships to the NL/1/2020-like genotype (Supplementary material). In contrast, a strikingly different phylogenetic pattern was observed for the PB2 segment (Fig 2), where the CZE/18520/2021 H5N1 virus was clearly separated from the NL/1/2020-like strains and showed preferential clustering with low pathogenic AI (LPAI) viruses, albeit with low statistical support. This suggests a much wider diversity of LPAI in nature. Taken together, the Czech H5N1 virus represents a novel 7:1 reassortant that retains the PB1-NS genomic cassette similar to NL/1/2020 supplemented with a distinct PB2 gene from unknown LPAI viruses presumably of Eurasian origin.

Analysis of the phenotypic characteristics showed that the CZE/18520/2021 H5N1 genome does not carry any critical mutations that confer antiviral resistance, enhanced replication capacity in mammals or preferential human receptor binding. Of particular interest is a naturally occurring alanine at position 156 (160 in H3 numbering) of the H5 HA molecule conferring increased binding to human-like receptors without loss of binding to avian-like receptors in the A/Vietnam/1203/2004 H5N1 virus backbone [14].

Available studies suggest that all H5N1 subtypes detected so far in Europe are monophyletic with a single circulating genotype similar to NL/1/2020 [3, 4]. NL/1/2020-like H5N1 viruses were first detected in October 2020 and were apparently generated by a 6:2 reassortment on an LPAI backbone with H5N8-like H5 and MP segments [4]. During 2021, the H5N1 subtype was reported mainly in the northern part of Europe and the UK, and it caused outbreaks in commercial poultry in Germany, the Netherlands, Slovakia and Hungary [3]. From May to September 2021, H5N1 was the most frequently detected HPAI subtype in wild birds in Europe [4]. Therefore, the sudden appearance of the H5N1 virus in a Czech poultry flock also suggests transmission from wild birds. Moreover, the farmer did not exclude the possibility that the poultry on the farm came into contact with wild waterfowl.

The identification of a new H5N1 genotype in the Czech Republic indicates ongoing genomic diversification in the wild bird reservoir in Europe. It can be assumed that the possibility of the H5N1 virus to re-assort increases even more in the autumn period, as its circulation coincides with the peak of LPAI in wild birds in nature [15]. In this regard the Saratov/2021-like H5N1 strain included in the phylogenetic trees suggests other recent genotype that were not the focus of our study.

The spread of H5Nx viruses during 2020-2021 resulted in one of the largest HPAI outbreaks ever recorded in Europe affecting more than 22.9 million poultry birds [3]. Fortunately, outbreaks in poultry have not been accompanied by human infections. However, the zoonotic potential of HPAI H5Nx viruses poses a permanent threat. This necessitates continuous surveillance both in avian and human populations.

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Table

Table 1. BLAST analysis of the CZE/18520/2021 H5N1 HPAI genome.

Segment	Strain	Acc. No.	Sequence identity
PB2	A/barnacle goose/Netherlands/2/2014 H3N6	EPI1010642	2246/2298 (97.7%)
PB1	A/chicken/Netherlands/20019879-001005/2020 H5N1	EPI1838671	2292/2299 (99.7%)
PA	A/common murre/Netherlands/21025491-002/2021 H5N1	EPI1859802	2182/2190 (99.6%)
H5	A/common murre/Netherlands/21025491-002/2021 H5N1	EPI1859803	1738/1742 (99.8%)
NP	A/chicken/Netherlands/20019879-001005/2020 H5N1	EPI1838674	1508/1513 (99.7%)
N1	A/common murre/Netherlands/21025491-002/2021 H5N1	EPI1859805	1419/1422 (99.8%)
MP	A/mute_swan/England/053054/2021 H5N1	EPI1924126	981/982 (99.9%)
NS	A/common murre/Netherlands/21025491-002/2021 H5N1	EPI1859807	846/850 (99.5%)

Given the high sequence identity between the CZE/18520/2021 H5N1 genomes, BLAST hits only for the A/goose/Czech_Republic/18520-1/2021 strain were shown.

Figures

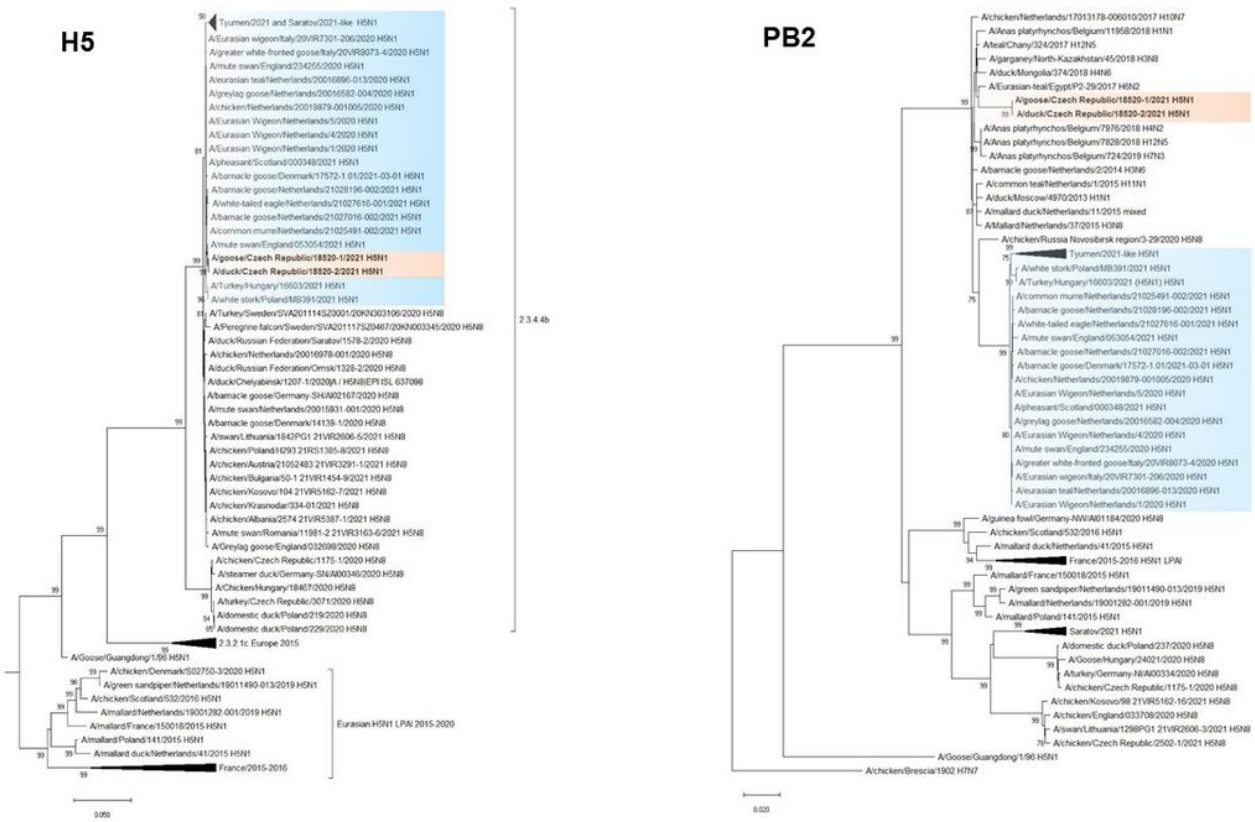

Figure 1

Phylogenetic analysis of the H5 and PB2 segments. Maximum-likelihood trees implementing the General Time Reversible model and discrete Gamma distribution of evolutionary rate differences between sites were constructed for representative avian influenza sequences obtained from the GISAID EpiFlu database. The codon positions included were 1st+2nd+3rd+noncoding. Bootstrap values (1000 replicates) in percentages were indicated at key nodes. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The H5 phylogenetic tree includes all available H5N1 sequences collected in Europe since 2015. The CZE/185202021 and NL/1/2020-like sub-clades were highlighted in blue and orange, respectively. Phylogenetic analysis of the remaining genomic segments is provided in the Supplementary material.

Supplementary Files

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