A new clade 2.3.4.4b H5N1 highly pathogenic avian influenza genotype detected in Europe in 2021

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Abstract
Despite their widespread distribution, only a single genotype variant of clade 2.3.4.4b H5N1 influenza viruses has been found so far in Europe. Here, we report the detection of a new highly pathogenic avian influenza H5N1 genotype in geese and ducks from a backyard farm in the Czech Republic. Phylogenetic analysis revealed that the Czech H5N1 virus retained the A/Eurasian_Wigeon/Netherlands/1/2020-like backbone with an altered PB2 segment obtained 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 evolutionary trajectory of the entire clade is characterized by extremely efficient global spread combined with high susceptibility to genetic reassortment [1]. Given the tremendous economic losses involved, thorough study of the molecular features and genotypes of emerging 2.3.4.4b H5Nx viruses is of paramount importance. The 2.3.4.4b H5Nx viruses were 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 multiple genotypes of the H5N8 and H5N5 subtypes have been identified, isolates of the H5N1, H5N3, and H5N4 subtypes have so far had the same genomic constellation [3, 4]. Here, 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 HPAI 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). The affected geese showed neurological signs of torticollis and uncoordinated movement. Veterinary inspectors immediately launched an investigation of the farm, took precautionary measures, and sent the dead birds to the 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 200 μl of pooled organ suspension using a MagNAPure Compact Total NA Extraction Kit (Roche) and eluted in 50 μl. RT-qPCR assays specific for generic influenza A virus, subtype H5, and cleavage site sequencing [6–9] revealed the presence of H5N1 HPAI. Subsequently, the remaining poultry on the farm were 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 from a goose specimen and a duck specimen was performed using 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 12.5 μl (10 μl of RT-PCR mix + 2.5 μl of total NA extract; primers available on request). 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.

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Basecalling was performed using Guppy, and demultiplexing and reference mapping were performed 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. Coinfection with other AI virus subtypes was excluded using neuraminidase-specific RT-PCR assays [10]. The consensus genomic sequences of two Czech H5N1/2021 strains (obtained from a goose, CZE/18520-1/2021, and duck, CZE/18520-2/2021) were submitted to the GenBank (accession nos. OL636392-99 and OL638145-52) and GISAID EpiFlu (accession nos. EPI1921673-88) databases. Phylogenetic analysis was performed using the MEGA10 program [11].

The two CZE/18520/2021 genome sequences were nearly identical, with two nucleotide transitions in the PB2, PA, and H5 segments and one in the PB1 segment. In addition, miscellaneous single-nucleotide variations were observed in segments PB2 (one in CZE/18520-1/2021 and two in CZE/18520-2/2021) and N1 (two in the CZE/18520-2/2021). The remaining segments, NP, MP, and NS, were identical at the nucleotide level. The nucleotide differences resulted in one amino acid change in PB2 (456S/N) and one in PA (65S/P).

The CZE/18520-1,2/2021 H5N1 strains had a multibasic amino acid motif PLREKRRKR*GLF at the cleavage site of the H5 HA gene that was identical to those of 2.3.4.4b strains [1], suggesting a highly pathogenic phenotype in chickens. A BLAST search of the GISAID EpiFlu database showed that all of the segments except PB2 of the CZE/18520-1,2/2021 H5N1 genomes showed ≥99.5% nucleotide sequence identity to those of recent H5N1 strains from the Netherlands and England. On the other hand, 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/18520-1,2/2021 sequences within the 2.3.4.4b clade and separation from previously circulating 2.3.2.1c and Eurasian LPAI H5N1 viruses [12–14] (Fig. 1). In addition, all H5N1 subtypes belonged to a single subclade, with A/Eurasian_Wigeon/Netherlands/1/2020 EPI_ISL_603133 (NL/1/2020) as the prototype, suggesting a monophyletic origin of the H5 segment. Similarly, dendrograms constructed for the other six 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. 1), where the CZE/18520-1,2/2021 H5N1 strains were clearly separated from the NL/1/2020-like viruses and showed preferential clustering with LPAI viruses, albeit with low statistical support. The absence of a clearly related PB2 sequence among known LPAI viruses and a low statistical

![Fig. 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.](image-url)
support for the critical branch also suggest that LPAI viruses have much greater diversity in nature than currently known. Taken together, the CZE/18520-1,2/2021 viruses represent novel 7:1 reassortants that retain the NL/1/2020-like PB1-NS genomic cassette but have acquired a distinct PB2 gene from unknown co-circulating LPAI viruses, presumably of Eurasian origin. Unfortunately, there is little surveillance of LPAI viruses in the Czech Republic, and therefore, the closest genetic relatives of the CZE/18520-1,2/2021 PB2 segment could not be identified.

Pairwise alignments of nucleotide and amino acid sequences of the CZE/18520-1,2/2021 and NL/1/2020 genomes (Table 1) revealed a high level of sequence identity at both the nucleotide and amino acid levels for the PB1-NS segments. As expected, the PB2 segment was strikingly different at the nucleotide level. Interestingly, this contrasted with the relatively high amino acid sequence similarity between the CZE/18520-1,2/2021 and NL/1/2020 PB2 proteins, suggesting a high frequency of synonymous mutations. This disparity correlates with the purifying selection acting on the PB2 protein (and other internal segments) that has been observed in AI viruses [14]. In addition, it also suggests a low level of constraint on PB2 segment exchange between NL/1/2020 and the unknown LPAI precursor of the CZE/18520-1,2/2021 genotype.

Analysis of phenotypic characteristics showed that the CZE/18520-1,2/2021 H5N1 genomes do not carry any critical mutations that confer antiviral resistance, enhanced replication capacity in mammals, or preferential binding to human receptors. Of particular interest is a naturally occurring alanine at position 156 (160 in H3 numbering) of the H5 HA molecule, which was shown to confer increased binding to human-like receptors without loss of binding to avian-like receptors in the A/Vietnam/1203/2004 H5N1 virus backbone [15].

Previous studies have suggested 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 HxN1 LPAI virus 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, according to the on-site investigation, the birds had direct access to an open water area.

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 likelihood of the H5N1 virus undergoing reassortment increases in the autumn, as its circulation coincides with the peak of LPAI in wild birds in nature [16]. In this regard, the Saratov/2021-like H5N1 strain, which was included in the phylogenetic analysis but was not the focus of our study, might also represent a new genotype.

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.

Table 1 Pairwise nucleotide and amino acid sequence identity values from comparisons of the CZE/18520-1,2/2021 and NL/1/2020 H5N1 HPAI genome sequences

| Segment/ gene | Genome   | Nucleotide sequence Differences | Identity | Amino acid sequence Differences | Identity |
|---------------|----------|---------------------------------|----------|---------------------------------|----------|
| PB2           | 18520/1  | 84/2298                         | 96.3%    | 4/759                           | 99.5%    |
|               | 18520/2  | 85/2298                         | 96.2%    | 6/759                           | 99.2%    |
| PB1           | 18520/1  | 9/2299                          | 99.6%    | 2/757                           | 99.7%    |
|               | 18520/2  | 10/2299                         | 99.6%    |                                 |          |
| PB1-F2        | 18520/1  | 3/273                           | 98.9%    | 2/90                            | 97.8%    |
|               | 18520/2  |                                 |          |                                 |          |
| PA            | 18520/1  | 13/2190                         | 99.4%    | 4/776                           | 99.5%    |
|               | 18520/2  | 5/776                           | 99.4%    |                                 |          |
| PAX           | 18520/1  | 7/759                           | 99.1%    | 1/252                           | 99.6%    |
|               | 18520/2  | 2/252                           | 99.2%    |                                 |          |
| H5            | 18520/1  | 4/1742                          | 99.8%    | 0/567                           | 100%     |
|               | 18520/2  | 6/1742                          | 99.7%    |                                 |          |
| NP            | 18520/1  | 6/1513                          | 99.6%    | 2/497                           | 99.6%    |
|               | 18520/2  |                                 |          |                                 |          |
| N1            | 18520/1  | 5/1422                          | 99.6%    | 2/469                           | 99.6%    |
|               | 18520/2  | 7/1422                          | 99.5%    | 4/469                           | 99.1%    |
| MP            | 18520/1  | 4/982                           | 99.6%    | –                               |          |
|               | 18520/2  |                                 |          | –                               |          |
| M1            | 18520/1  | 4/759                           | 99.5%    | 0/252                           | 100%     |
|               | 18520/2  |                                 |          |                                 |          |
| M2            | 18520/1  | 0/293                           | 100%     | 0/97                            | 100%     |
|               | 18520/2  |                                 |          |                                 |          |
| NS            | 18520/1  | 4/850                           | 99.5%    | –                               |          |
|               | 18520/2  |                                 |          | –                               |          |
| NS1           | 18520/1  | 4/693                           | 99.4%    | 2/230                           | 99.1%    |
|               | 18520/2  |                                 |          |                                 |          |
| NS2           | 18520/1  | 2/366                           | 99.5%    | 1/121                           | 99.2%    |
|               | 18520/2  |                                 |          |                                 |          |
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Author contributions AN conceptualized the study and wrote the original draft, participated in data curation, and conducted next-generation sequencing and phylogenetic analysis. LC performed virus detection and pathotyping and was involved in data curation and manuscript preparation. MS participated in virus detection, pathotyping, sequencing library preparation, data curation, and manuscript preparation.

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Data availability The consensus genomic sequences of the two Czech H5N1/2021 (CZE/18520/2021) strains were submitted to the GenBank (Accession Nos. OL636392-99 and OL638145-52) and GISIAD EpiFlu (Accession No. EPI1921673-88) databases.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This article does not contain any studies with animals performed by any of the authors. The ethical standards of animal welfare are under the supervision of the State Veterinary Administration of the Czech Republic.

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