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Case report

FIRST CASE OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 IN MONTENEGRO

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Avian influenza, commonly known as bird flu, is a highly infectious viral disease that affects birds, including wild water birds and poultry. The emergence and spread of highly pathogenic avian influenza (HPAI) strains, such as H5N1, have raised concerns due to their potential to cause severe outbreaks and cross the species barrier, leading to human infections and global public health emergencies. In this study, we report the first case of HPAI H5N1 detection in Montenegro. Twenty-six carcasses of dalmatian pelicans were found in Skadar Lake, Montenegro, and the H5N1 subtype was confirmed through molecular testing in the samples from pelicans. The whole influenza genome was sequenced and belonging to clade 2.3.4.4b was determined.

Keywords: 2.3.4.4b, Avian influenza, HPAI, H5N1, Montenegro

INTRODUCTION

Avian influenza, commonly known as bird flu, is a highly infectious viral disease that primarily affects birds, particularly wild water birds and poultry [1]. The virus has gained significant attention from scientists, public health experts, and policymakers due to its potential to cause severe outbreaks with serious implications for animal health, agriculture, and human health. The emergence and spread of highly pathogenic avian influenza (HPAI) strains have raised concerns about their potential to cross the species barrier and cause more human infections, leading to global public health emergencies [2].

Avian influenza is caused by influenza A viruses, belonging to the Orthomyxoviridae family. These viruses possess a unique ability to undergo rapid genetic mutations, leading to the emergence of new strains with varying degrees of pathogenicity. The avian influenza virus predominantly circulates among wild birds, which act as natural

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reservoirs. However, when it spills over into domestic poultry populations, it can have devastating consequences.

In the latter part of 2020, various genotypes of Highly Pathogenic Avian Influenza (HPAI) belonging to clade 2.3.4.4b emerged in wild bird populations. H5 genotypes can spread rapidly among poultry flocks, causing high mortality rates and significant economic losses within the agricultural industry, for example, H5N8 outbreak in 2020 resulted in loss of over 70 million birds [3]. Although the H5N8 virus responsible for the 2020-2021 outbreaks in Eurasia and Africa exhibited genetic stability with the 2.3.4.4b genotype, the emergent 2.3.4.4b H5N1 viruses in the current panzootic context appear to have loosened constraints on extensive genetic reassortment [4]. This adaptation suggests an increased potential for enhanced transmission and sustained existence at the interface between domestic and wild bird populations.

While the contemporary H5N1 virus is indeed not crossing into human hosts as frequently as it did before 2016, it demonstrates a heightened aptitude for long-distance transmission via migratory and wild birds [5]. The HPAI H5N1 clade 2.3.4.4b is now the most common strain responsible for global avian influenza outbreaks [6]. It has rapidly disseminated on a global scale and continues to explore new potential mammalian hosts. Since September 2022, A(H5) viruses have been detected in both domestic and wild birds in many countries, with sporadic detections in mammals.

A crucial role in the ability of influenza viruses to spread and cause infections is played by a proteolytic cleavage of the virus surface glycoprotein, the hemagglutinin (HA) [7]. In low pathogenicity avian influenza viruses (LPAIVs), the specific sequence at which haemagglutinin is cleaved (referred to as the haemagglutinin cleavage site or HACS motif) usually holds one or two basic amino acid residues that are not consecutive. These residues are cleaved by enzymes like trypsin and trypsin-like proteases with monobasic specificity [8]. Thus, the replication of LPAIVs is generally limited to cells in the respiratory and gastrointestinal tracts that express trypsin.

In stark contrast, the HACS motif of highly pathogenic avian influenza viruses (HPAIVs) comprises multiple basic amino acids which facilitate cleavage by proteases that are widely expressed throughout the body, with the most notable one being furin, which has a polybasic specificity [9]. This adaptation allows highly pathogenic viruses to replicate in various types of tissues, for example, with a particular inclination to reproduce in the vascular endothelium of chickens [10]. This enables highly pathogenic viruses to replicate in various types of tissues, with a preference for the inner lining of blood vessels in chickens. This particular preference for blood vessels is considered a major reason why these viruses are so deadly in this species.

Efforts to monitor and control avian influenza outbreaks have become a global priority. Surveillance programs, early detection systems, and strict biosecurity measures have been implemented to prevent the spread of the disease among poultry and minimize the risk of human infections. Some countries affected by the endemic presence of HPAI H5 engage in regular poultry surveillance, while others primarily collect samples

in response to large-scale bird mortality incidents. The sampling of wild birds is accorded lower precedence and frequently exhibits a bias towards species that are readily accessible for sampling. Notably, despite the preferential focus on poultry in the sampling process, some findings indicate that the spread of clade 2.3.4.4b H5 viruses by wild birds is comparable to that of domestic birds, which were previously presumed to be the principal catalysts of HPAI H5 dissemination [11].

The development of influenza candidate vaccine viruses (CVVs) for production and distribution of effective vaccines for both animals and humans, coordinated by WHO, remains an essential component of the overall global strategy for influenza pandemic preparedness [6].

The first case of High Pathogenic Avian Influenza in Montenegro (H5N5)

The first case of High Pathogenic Avian Influenza (HPAI) in Montenegro was reported in the winter of 2016. The case involved a mallard and was detected through active surveillance [12]. The specific subtype was H5N5, and no clinical signs were observed. During that same period, several European countries, including Croatia [13], the Netherlands [14] and others [15] also reported H5N5 infections in wild birds. However, until now, Montenegro has not reported any cases of the H5N1 subtype of HPAI.

In 2022, all neighboring countries of Montenegro reported cases of HPAI either in domestic poultry or in wild birds. Croatia reported cases during the winter season of 2021-2022, affecting both domestic poultry and wild birds, according to data from the Ministry of Agriculture of Croatia. Additionally, in Serbia during November of 2021, the H5N1 strain was detected in swans [16].

The first case of High Pathogenic Avian Influenza in Montenegro (H5N1)

Here we report about the first case of HPAI, H5N1 detection in Montenegro. Montenegro conducts active and passive surveillance of the presence of HPAI in wild birds. During the winter season 2021-2022, no cases of HPAI were identified in Montenegro, and there were no reported instances of mortality among wild birds at Skadar Lake. Skadar Lake is the largest and most significant wetland in Montenegro. It has been designated as a Ramsar site and an important bird area due to the substantial numbers of migratory and breeding water birds it hosts. The lake serves as a crucial stopover site within the Black Sea – Mediterranean flyway for numerous populations of migratory water birds, including Dalmatian pelicans. Currently, it marks the westernmost point of the Dalmatian pelican's breeding range, with documented exchanges of this species' population occurring with colonies in Southeast Europe [17]. However, in early April 2022 it was reported five carcasses of Dalmatian pelicans in Skadar lake. First positive case was confirmed on April 7th at the Diagnostic Veterinary Laboratory in Podgorica. The total number of 26 carcasses of Dalmatian pelicans were found in Montenegrin part of Skadar lake in the nesting area of Panceva Oka.

The aim of the study was to investigate and document the first case of HPAI H5N1 in Montenegro. The study aims to provide an account of the outbreak, obtain the sequence of the virus from this outbreak, assess the pathogenicity of the virus, and determine its clade.



Figure 1. Scheme of Skadar Lake with pointed Pancheva Oka – place of infected pelicans' discovery.

METHODS

Sample collection and necropsy

A total of 13 carcasses out of 26 were delivered to the Diagnostic Veterinary Laboratory. Carcasses were delivered on three occasions during April 2022. A necropsy was performed on four delivered dead pelicans. The necropsy was performed in the necropsy room of the Diagnostic Veterinary Laboratory. Delivered carcasses were fresh. The necropsy was performed using all necessary biosecurity measures.

RNA extraction

When carcasses arrived at the laboratory, cloacal and tracheal swabs were taken in the necropsy room (from all 13 animals) as well as parts of the lungs and trachea. RNA extraction was performed manually using High Pure Viral Nucleic Acid Kit (Roche). The extraction of RNA followed protocols recommended by the manufacturer.

PCR setup

After RNA extraction, preparation of PCR mix started. For amplification QuantiTect Probe RT PCR kit was used. The first step was detection of M gen (Spackman et al, 2003) [18] and after that detection of H5 (Slomka et al, 2007) [19], H7 [20] and N1 [21] subtypes of avian influenza virus. After the subtype detection, NGS was undertaken.

Sample sequencing library preparation and nucleotide sequencing

The virus genome segments from the sample were amplified using the pan-influenza primers [22] and the Superscript III One-Step RT-PCR with Platinum Taq Reagents kit (Invitrogen, Thermo Fisher Scientific, Massachusetts, United States). The library for sequencing was prepared using a Rapid Sequencing Kit (SQK-RAD004) (Oxford Nanopore, Oxford, UK) and subsequently loaded onto a R9.4.1 flowcell (FLO-MIN106) according to the manufacturer's instructions. Sequencing and data acquisition were performed using a MinION Mk1C (Oxford Nanopore, Oxford, UK); the run lasted for six hours, with high-quality basecalling being done in real time.

Genome Assembly and Consensus Correction

Guppy software was used for base-calling and data quality trimming. FastQC software was used for sequence data quality assessment. Trimmomatic was applied for quality data trimming. Reads were mapped onto the reference H5N1 influenza sequence using Minimap2 [23]. SAMtools-mpileup v1.6 [24] was used to collate the reads at each genomic position, then iVar v1.3 [25] was used to call the consensus sequence based on the mpileup output.

Phylogenetic analysis of generated sequence

The annotation of mutations was performed using the GISAID FluServer tool [26]. Sequences for the dataset were downloaded from GISAID database [26]. Dataset included sequences from different 2.3.4.4 clades from WHO dataset for "Genetic and antigenic characteristics of zoonotic influenza A viruses and development of candidate vaccine viruses for pandemic preparedness" [1], animal H5N1 sequences, human H5NX sequences and rooted on clade 2.3.4 sample (A/Anhui/1/2005). Phylogenetic tree was created from dataset by MEGA [27] using Neighbor-joining tree method and then visualized by iTol v6 [28].

RESULTS

In April 2022 a total 26 carcasses of Dalmatian pelicans was found in the Montenegrin part of Skadar Lake, specifically in the nesting area of Panceva Oka. The pelican carcasses were fresh and in good condition, showing no signs of mechanical or

gunshot injuries, and there was no evidence of bleeding from natural openings. Pathomorphologically, intense bleeding was found on the pericardium, pancreas, air sacs, mesentery and intestines. There was no bleeding in the trachea and proventriculus. A small amount of serous content was present in the trachea and lungs. Samples taken from all 13 delivered pelicans were positive on the presence H5N1 subtype of HPAI. The first positive case was confirmed on April 7th at the Diagnostic Veterinary Laboratory in Podgorica.

After detecting the subtype, the entire influenza genome was amplified. Samples that yielded the best amplification results underwent sequencing using the Oxford Nanopore MinION platform. Genome was assembled without any gaps and the assembled genome was uploaded to the GISAID database under the name A/pelican/Montenegro/833/2022 and was assigned the identifier EPI_ISL_17731667.

Sequence data analysis

The clade of the sequenced sample was defined as 2.3.4.4b. HACS motif is known [29] flanked N-terminally by PQ/L and C-terminally by GLF sequences. In the influenza virus genome sequence obtained the following the cleavage site was observed PL_REKRRKR↓GLF and classified as highly pathogenic. None of the segments have 100% amino acid identity with the references in the database. The list of mutations is provided below (Table 1).

Table 1. List of mutations in the resulting sequence

Gene	Mutations list
HA	K3N, G16S, N110S, T139P, T156A, Q185R, V194I, A201E, N252D, E284G, M285V, I298V, K492E, V538A, I547M, V548I
NA	K6R, I10T, V17I, I20V, H44Y, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, T289M, G336S, V338M, P340S, N366S, G382E, S405T, I418M, S434N, D460G
M1	L55M, T140A, F144L, M165I, K230R, N232D, M248L
M2	R12K, K18N, I51V
NP	M105V, V186I
NS	S83P, L147I, D171N, V226I, E67G
PA	T85A, G114E, D160E, I201T, K269R, E300R, G301del, I302del, P303del, L304del, Y305del, D306del, I308Y, K309R, V322L, I354F, K391R, V432I
PB1	N16D, T117A, E172D, K176R, R214K, R430K, K635R, N694G
PB2	I66M, V338I, I478V, R664K

Many recognized mutations that are not found in the sequences used to derive the mutation statistics at the GISAID database have equivalent mutations in resolved structures of proteins from related strains. Some of these mutations could play a role in the viral life cycle, such as the example of N694G in PB1, which is involved in binding viral proteins.

The G16S polymorphism in hemagglutinin protein plays a role in the immune escape, as it is located within a T-cell epitope presented by MHC molecules [30] and antibody recognition sites [31]. Additionally, the mutations N110S and T139P in HA are associated with a shift in host specificity [32,33] and changes in antibody recognition sites [34,35]. The mutation equivalent to T156A in HA [36] is related to antigenic drift, leading to the emergence of an escape mutant. Furthermore, several other mutations found in HA, including A201E [37], E284G and E285M [38], K492E [39] are located within antibody recognition sites.

There are also some important mutations in neuraminidase. The H155Y substitution is associated with a strong resistance to Tamiflu and Relenza [40]. H155Y is also located in antibody recognition sites, as well as V338M, N366S and S434N mutations [41,42]. A mutation at position A369D which is equivalent to N366S in obtained sequence (the numbering may vary among different strains) has been reported [43] to cause antigenic drift.

Two mutations are associated with an increase in virulence in NS proteins: V226I in NS1 and E67G in NS2. It has been suggested that the E67G substitution in NS2 protein may impact the replication of all three polymerase genes in the viral genome, leading to the generation of defective interfering particles of the virus [44]. The V226I in NS1 also can affect virulence [45].

Phylogenetic relatedness with H5N1 viruses

To investigate the genetic relationships of the Montenegrin HPAI H5N1 sample isolated in this study, we conducted phylogenetic analyses using representative strains of avian influenza virus. These analyses supported the genetic findings and demonstrated that the Montenegrin HPAI H5N1 strains were closely associated with H5N1 strains observed in Europe, Asia, and Africa between 2021 and 2022.

Specifically, the HA and NA gene segments of the A/pelican/Montenegro/833/2022 Montenegrin HPAI H5N1 isolate exhibited close phylogenetic proximity to variants detected in Russia, Estonia, and Slovakia, belonging to clade 2.3.4.4b (Figure 2). Notably, the phylogenetic analysis of the PB2, PB1, PA, NP, M, and NS gene segments revealed distinct clustering patterns, indicating evidence of multiple reassortments within the H5N1 strains of clade 2.3.4.4b, as previously described [46].

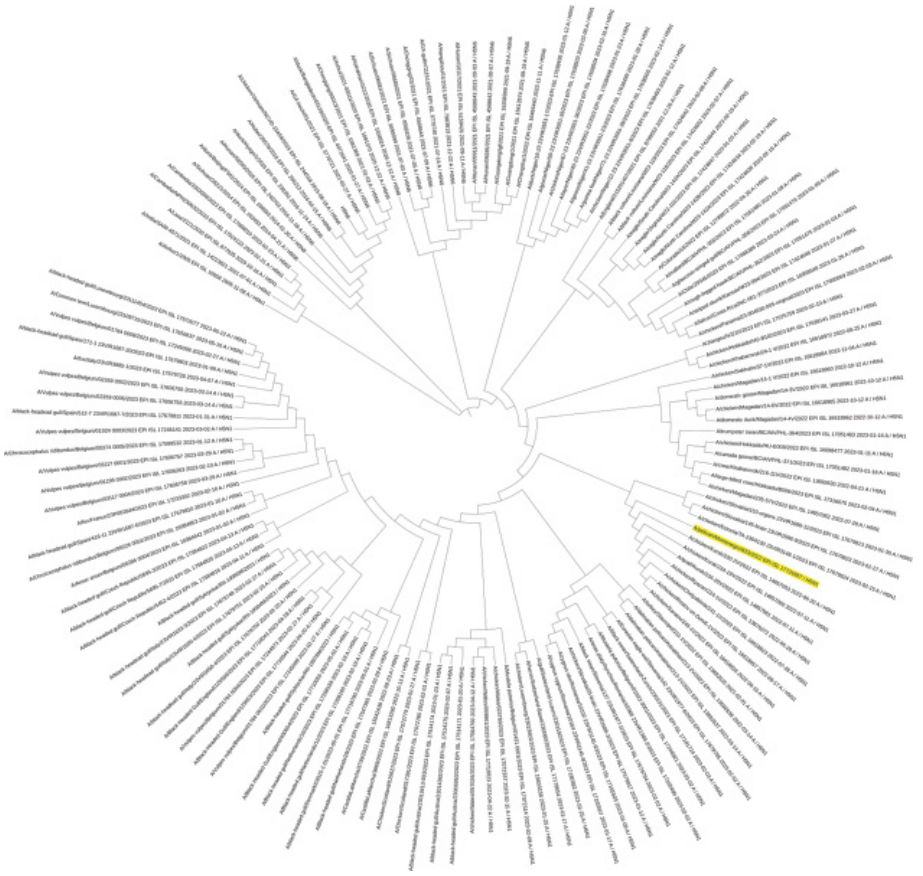


Figure 2. Neighbor-joining phylogenetic tree of H5 sequences. Phylogenetic tree of the nucleotide sequences of the HA gene segments of the avian influenza H5 subtype. The obtained sequence position is highlighted in yellow.

DISCUSSION

In the 2021-2022 epidemiological year, Europe experienced the largest High Pathogenic Avian Influenza (HPAI) epidemic to date, with a total of 6,615 detections of HPAI virus across 37 countries [47]. What was particularly unexpected was the continued spread of the epidemic throughout the summer months, affecting colony-breeding seabirds, a group of species that had rarely been reported as affected by HPAI before. Among the seabirds, the Peruvian pelican stood out as the most frequently infected species, with thousands of deaths reported [33].

Here, we also present findings regarding pelicans that have succumbed to infection with HPAI. Pelicans are known to be highly susceptible to viral infections due to their tendency to reside in densely populated colonies [48]. The first case of High Pathogenic Avian Influenza (HPAI) H5N1 in Montenegro is presented in this report.

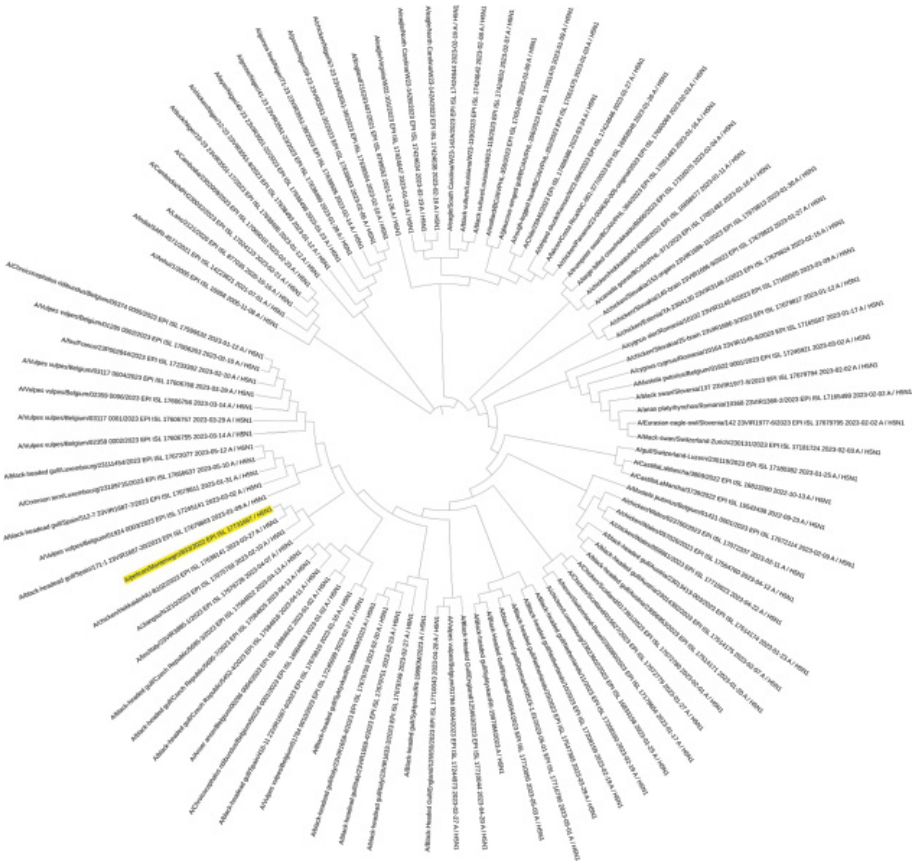


Figure 3. Neighbor-joining phylogenetic tree of N1 sequences. Phylogenetic tree of the nucleotide sequences of the NA gene segments of the avian influenza N1 subtype. The obtained sequence position is highlighted in yellow.

Until April 2022, no cases of HPAI were identified in Montenegro and there were no reported instances of mortality among wild birds at Skadar Lake.

The detection of avian influenza is a crucial aspect of response and prevention in controlling the spread of the virus. Sequencing avian influenza genomes enables the development of the most appropriate candidate vaccine viruses for influenza. It provides new data on the epidemiology of avian influenza and contributes to our understanding of its genetic diversity. The virus that caused the outbreak in Montenegro belongs to clade 2.3.4.4b and have the HACS motif of highly pathogenic avian influenza viruses as determined by our sequencing results. Clade 2.3.4.4b is related to HPAI viruses, emerged in 2014 [49] and have been associated with multiple outbreaks in different regions. The exact origin of this clade is not well-defined, but it is believed to have originated from the reassortment of different avian influenza strains [6].

The first case of highly pathogenic avian influenza in Montenegro reinforces the importance of effective communication and coordination among government agencies, veterinary services, public health authorities, and other stakeholders involved in disease management. In addition to avian populations, mammals have also been implicated in influenza transmission, as they prey upon infected birds. Since October 2020, reports have surfaced involving 24 carnivore species, 4 cetacean species, as well as domestic pigs, wild boar, and Virginia opossums [33]. Building public awareness about avian influenza, its potential risks, and the necessary precautions is essential for minimizing human exposure and preventing potential outbreaks.

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Authors' contributions

BA conducted molecular analysis and prepared samples for sequencing, coordinating all tasks related to samples and publication preparation. SG performed sample sequencing and participated in obtaining the sequence consensus. NP conducted a necropsy on bird specimens. AV discovered decreased birds, providing the laboratory with bird carcasses. NY processed sequencing data, participated in obtaining the sequence consensus, analyzed the sequence, and drafted the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PRVI SLUČAJ VISOKO PATOGENE AVIJARNE INFLUENCE H5N1 U CRNOJ GORI

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Avijarna influenca, poznata i kao ptičji grip, je visoko zarazna virusna bolest koja pogađa ptice, uključujući divlje vodene ptice i domaću živinu. Pojava i širenje sojeva visoko patogene ptičje gripe (HPAI), kao što je H5N1, izaziva zabrinutost zbog njihovog potencijala da izazovu ozbiljne epidemije i pređu barijeru vrsta, što potencijalno dovodi do infekcija ljudi i globalnih vanrednih situacija po javno zdravlje. U ovoj studiji izveštavamo o prvom slučaju detekcije visokopatogene avijarne influence H5N1 u Crnoj Gori. Na Skadarskom jezeru, u Crnoj Gori, pronađeno je 26 leševa dalmatinskih pelikana, a podtip H5N1 potvrđen je molekularnim ispitivanjem u uzorcima poreklom od pelikana. Celokupni genom virusa gripa je sekvencioniran i određena je pripadnost klasteru 2.3.4.4b.