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OUTBREAK OF INFLUENZA A VIRUS (H5N1) IN DALMATIAN PELICANS SREBARNA RESERVE, BULGARIA, 2015

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ABSTRACT

On March 25, 2015, a highly pathogenic avian influenza viruses were isolated from the carcasses of a 3 Dalmatian pelicans (Pelecanus crispus) in Bulgaria. Polymerase chain reaction (PCR) and H5N1-specific real-time reverse transcription polymerase chain reaction (rRT-PCR) analysis showed, that the H5 and N1 avian influenza virus was presented in the lung, trachea, proventriculus, cloaca and brain tissue of the dalmatian pelicans. Subsequent sequence analysis found the following motif of basic amino acids at the cleavage site of hemagglutinin: PQRERRRKRGLF, which is characteristic of highly pathogenic avian influenza viruses. Phylogenetic analysis of a part from segment 4 of A/dalmatian pelican/ Sreburna/Bulgaria/2015 (H5N1) showed a close genetic relationship with influenza viruses A (H5N1) clade 2.3.2.1. The establishment of HPAI H5N1 in 2015, belonging to the genetic clade 2.3.2.1, circulating in Southeast Asia and in Bulgaria for the second time since 2010, shows the great potential for the trans-continental distribution of the virus and its ability to cause not only epizootic outbreaks, but also panzootic waves.

Keywords: H5N1, Bulgaria, Dalmatian pelicans, Phylogenetic analysis

INTRODUCTION

Influenza A infections are among the most dangerous and significant illnesses in many species of animals and people. Their zoonotic potential has always inspired a great interest among scientists and not once terrible fear among the populations of the world. Avian Influenza is particularly crucial since waterfowl migratory birds are the main reservoir and vector of infection. All influenza viruses except H17N10 and H18N11, which are reportedly found in bats in Guatemala (Tong et al., 2012), are detected in waterfowl birds. Naturally occurring infections with avian influenza viruses (AIVs) have been reported in free-living birds from 26 families, representing 105 species (Olsen et al., 2006). They can emit virus 30 days after infection, which is a precondition for its dissemination over long distances during migration of birds and introduction into populations of domestic birds. Based on their ability to cause clinical disease with high mortality in domestic chickens, influenza strains are divided into high pathogenic (HP), and low pathogenic (LP) subtypes. So far, naturally occurring viruses that cause HPAI have been associated with subtypes H5 and H7. However, H5 and H7 viruses are also presented in LP forms (Alexander, 2007).

AIVs are classified as HP for poultry, when the intravenous pathogenicity index (IVPI) in six-week-old chickens is either greater than 1.2 or causes at least 75% mortality in four-to-eight-week-old chickens infected intravenously. The same classification is applied when the characteristic motif of basic amino acids in the cleavage site of HA (PQRESRRKK/GLF) is identified after sequence analysis (OIE Terrestrial Manual, Chapter 2.3.4, 2015).

The occurrence of H5N1 in Bulgaria was first reported in 2006 in swans and geese (Goujgoulova and Oreshkova, 2007). In 2010, H5N1 was reported in common buzzard (Marinova-Petkova et al., 2012), and in 2015 in dalmatian pelicans, pigeon and black-headed gull (1 REF OIE 17083, World Organisation for Animal Health (OIE), 2015).

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On the 25th of March 2015, virus isolation (VI) confirmed that H5N1 caused the death of 21 dalmatian pelicans in the Srebarna reserve. Several days later, over 100 dalmatian pelicans from the Danube Delta in Romania were also found dead. Furthermore, during 2015, HPAI H5N1 had been reported in Russia, Bulgaria, Romania and Kazakhstan (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/). In 2015 avian influenza in Bulgaria was announced also in rock dove (Columbia livia), the black-headed gull (Chroicocephalus ridibundus) and in domestic poultry (backyard farm) village Konstantinovo, Burgas municipality.

MATERIALS AND METHODS

Collection of samples. A total of 21 recently perished dalmatian pelicans at the Srebarna reserve were collected for the study. However, dalmatian pelicans showed advanced stages of putrefaction and we were able to sequence 3 viruses.

Virus isolation and identification. Organs from each animal were pooled, homogenized, and the samples were centrifuged at 800g for 10 minutes at 4°C. Inoculation into the allantoic cavity of three 10-day-old embryonated chicken eggs (ECE) was performed using 200µl of supernatant from each organ sample. The infected embryos were incubated at 36°C for up to 120 hours and were checked every day during the test. All chicken embryos were found dead after 24-48 hours and their allantoic fluids were tested for hemagglutination activity via the hemagglutination assay (HA assay). The HA positive allantoic fluids were examined for hemagglutination inhibition (HI) using 4 hemagglutination units per well and hyperimmune standard serum (H5N1, H5N3) produced by Instituto Zooprofilattico delle Venezie (Comin et al., 2013; Molesti et al., 2014). The standard OIE procedure was followed for both the HA and HI assays (OIE Terrestrial Manual, Chapter 2.3.4, 2015).

Nucleic acid detection and sequence analysis. Viral ribonucleic acid (RNA) was extracted from virus-containing allantoic fluid by using QIAamp Viral RNA Mini kit (Qiagen) following manufacture protocol. The oligonucleotide primers (table 1) were used to amplify a 300-320 base pair fragment of the HA protein, which includes the HA cleavage site (Slomka et al., 2007). After that, the positive samples were purified with a purification kit Wizard® SV Gel and PCR Clean-Up System (Promega) with a centrifugation according the standard procedure. Purified product at a concentration of 10ng/µl was sent for sequencing to LGC GENOMICS Germany.

Table 1. Primer used to amplify 300-320 base pair fragment which include HA cleavage site.

AIV_06_H5 kha 1-F:	**5' CCT CCA GAR TAT GCM TAY AAA ATT GTC 3'**
AIV_06_H5 kha 3-R:	**5' TAC CAA CCG TCT ACC ATK CCY TG 3'**

Phylogenetic Analysis. We performed a phylogenetic analysis of a part from segment 4 of A/dalmatian pelican/ Sreburna/Bulgaria/2015 (H5N1). The processing sequence and the preparation of phylogenetic trees were completed with the help of BioEdit, Staden, MEGA 7.0 and NCBI (National Center for Biotechnology Information) software. Identification of the amino acid motif at the cleavage site of HA to establish the pathogenicity of strains was performed using the program BioEdit. The alignment of the sequences was performed by the ClustalW method in the BioEdit program. In the construction of phylogenetic trees, the Neighbor-Joining method, (maximum composite likelihood model) was implemented. We applied the Bootsrap method (probability confidence) using 500 bootstrap replicates in order to confirm the topography of the branches of the phylogenetic trees. A/bar-headed goose/Mongolia/X25/2009 (H5N1) and A/Anas acuta/Slovenia/470/06 (H5N1) viruses obtained from the influenza virus research database were used as reference strains for clades 2.3.2.1 and 2.2.1 respectively.

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RESULTS

Virus isolation and identification. Pooled samples of lungs, brain, small intestine, proventriculus, trachea and cloaca tissues from Dalmatian pelicans were injected into ECE and an avian influenza virus (*A / dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1)*) was isolated. The isolate was subjected to hemagglutination inhibition (HI) assays, as specified in the OIE Terrestrial Manual, Chapter 2.3.4, 2015, and was defined as H5 subtype. We isolated 3 HPAIVs by virus isolation in ECE from 3 dalmatian Pelicans, found dead in the Srebarna reserve.

Nucleic acid detection and sequence analysis. Polymerase chain reaction (PCR) and H5N1-specific real-time reverse transcription polymerase chain reaction (rRT-PCR) analysis showed that the H5 and N1 AIV was present in the lung, trachea, proventriculus, cloaca and brain tissues of the dalmatian pelicans. RT-PCR was performed on the viral RNA using AIV-specific oligonucleotides, and the HA cleavage site was sequenced in order to determine the pathotype (Fig.1). Subsequent sequence analysis found the following motif of basic amino acids at the cleavage site of hemagglutinin: PQRERRRKRGLF (Fig. 2), which is characteristic of HPAIVs (Horimoto et al., 1994).

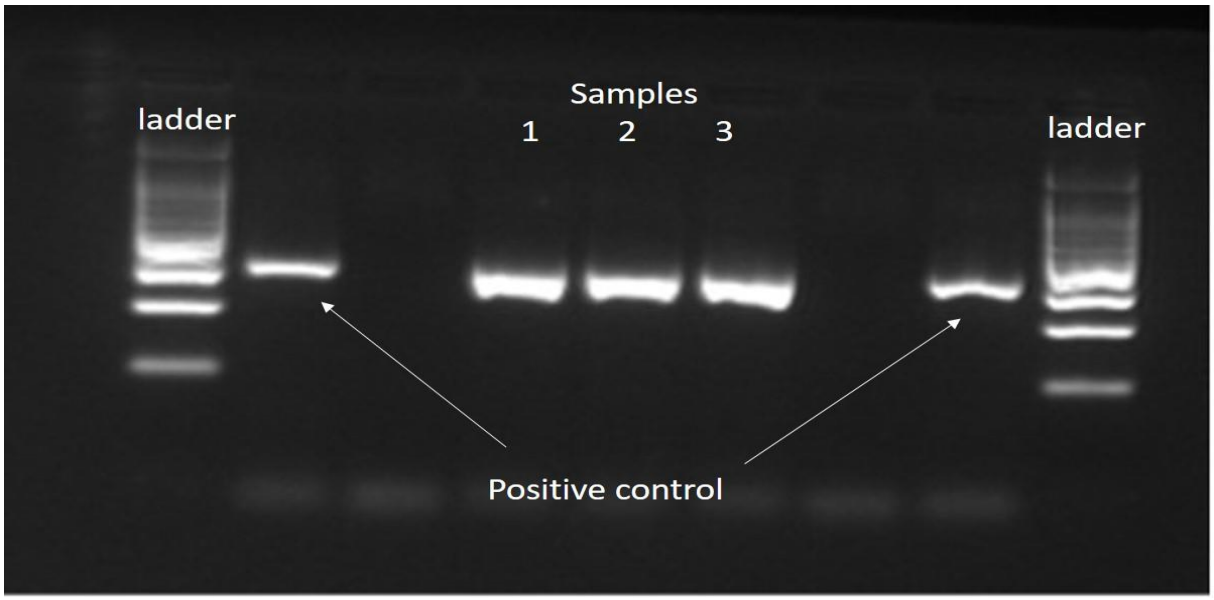

Fig.1. Gel electrophoresis visualizing positive samples when using primers KHA.

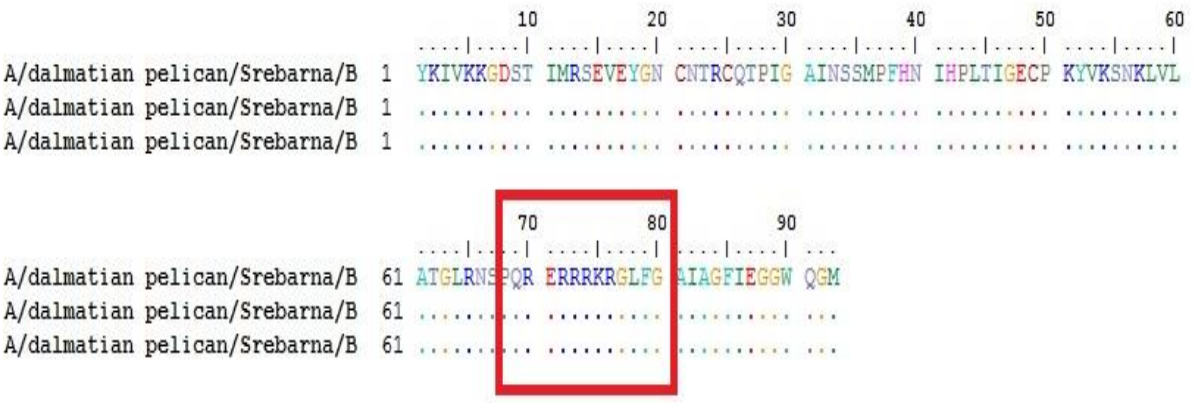

Fig.2. Comparison of AA sequences of three isolates A/dalmatian pelican/ Sreburna/Bulgaria/2015 (H5N1). Red rectangle marks AA sequences at the cleavage site of the HA.

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Phylogenetic analysis.

Because of, until 2015, only two H5 clades had demonstrated transcontinental propagation ability, we decided to compare virus isolates of dalmatian pelicans with isolates obtained so far in Bulgaria, while using reference viruses from clades 2.3.2.1 and 2.2.1. A / dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1) were clustered with 2.3.2.1 viruses by forming a separate cluster (Fig 3).

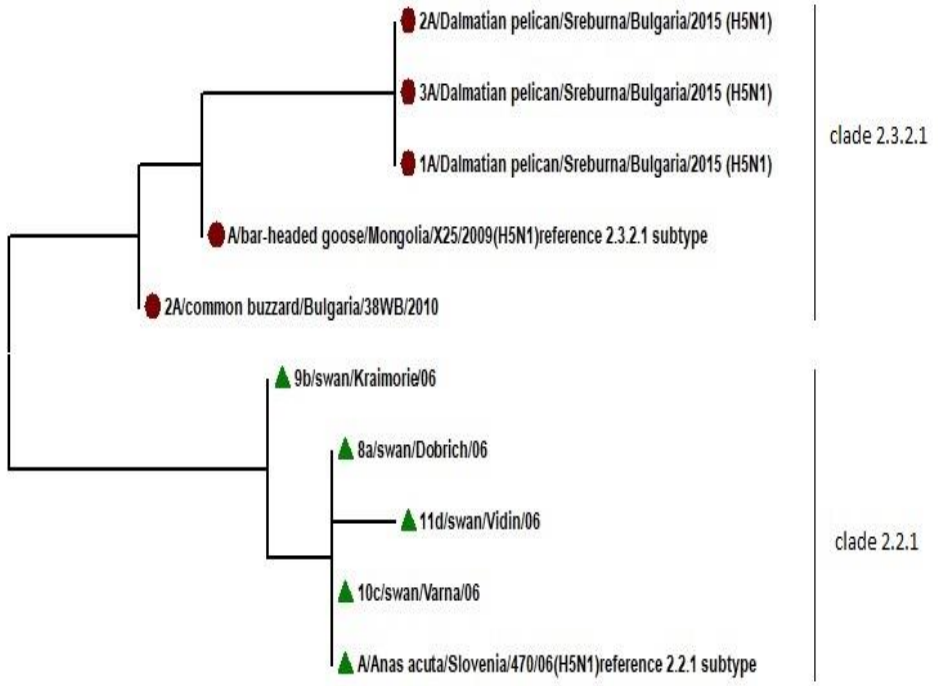

Fig. 3. Phylogenetic analysis: comparison between the Bulgarian isolates 2006, 2010 and 2015 including reference viruses from clades 2.3.2.1 and 2.2.1. With red circle are marked viruses belongs to clade 2.3.2.1, with green triangle those from 2.2.1.

DISCUSSION

In general, the main direction of the migrating patterns of pelicans over the territory of Bulgaria is north - south or south - north depending on the direction of migration (autumn versus spring). Movements are mainly from the Danube Delta to the Bosphorus and the Mediterranean Sea and vice versa respectively. It is also probable that the virus has reached Romania in this way. The hypothesis that the virus spread throughout Bulgaria and Romania through this migratory patterns is supported by the results of the phylogenetic analysis and the comparison of the segment 4 (HA) of the isolates from Bulgaria and Romania (Tassoni at al., 2016). After sequential analysis and the establishment of the genetic subtype, we can now assume with greater certainty where the virus has come from and how it has spread to Bulgaria, Europe and Africa (Tassoni at al., 2016). The phylogenetic analysis clustered Vietnamese H5N1 isolated in 2012 and 2013, belonging to subtype 2.3.2.1c. These results undoubtedly suggest that the virus is of Asian origin.

Most of the wild birds affected with the H5N1 strain of HPAI are Pelicanus crispus. This bird species migrates over short distances, and even repeated overlaps of its migration routes and habitats cannot explain the genetic origin of the circulating strain of this genetic subtype in Europe,

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without evidence of its detection on migratory routes through Central Asia in other long distance migrating birds. Within its detection at the Altay region of (Siberia - Russia) from 1 pelican (27.04.2015) and in Kazakhstan of 2 pelicans (22.05.2015), the puzzle begins to be stacked (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/). Isolated viruse from these two regions belongs to the same clade 2.3.2.1c and have 98.83% and 98.44% sequence identity in a 258 bp sequence of the HA gene with Influenza A / Guandong / 1/96 lineage virus, subtype 2.3.2.1, as viruses from China and other viruses from Vietnam, isolated in the period 2012-2014 in Southeast Asia and India. In May 2015, the HPAI virus of the H5N1 strain with the genetic subtype 2.3.2.1.c continued to cause epizootic outbreaks in Turkey. One dead Pelican was found on the Black Sea coast in a reserve in the area of Kastamonu (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/).

On May 20, 2015, there was another, third outbreak of the HPAI H5N1, affecting a herd of 170,000 birds in the Manisa region, which is characterized by a high concentration of poultry farming in Turkey (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/). After its emergence in Europe in the first months of 2015, HPAI H5N1 was observed, with subtype 2.3.2.1c, being identified in Africa, namely in the Ivory Coast, Burkina Faso, Niger and Ghana. The viruses isolated there were phylogenetically similar to those isolated in Bulgaria and Romania. Phylogenetic analysis of the isolates from these countries found that they form two different branches, one of them (W2) being closer to the Bulgarian and Romanian isolates (99.3% and 99.65% respectively). These results indicate that the virus may have entered Africa via two different routes (Tassoni et al., 2016).

CONCLUSION

In conclusion, the establishment of HPAI H5N1 in 2015, belonging to the genetic clade 2.3.2.1, circulating in Southeast Asia and in Bulgaria for the second time since 2010, shows the great potential for the trans-continental distribution of the virus and its ability to cause not only epizootic outbreaks, but also panzootic waves. The fact that it has infected people in Asia poses a particular challenge to public health authorities. The rapid geographic spread of highly pathogenic avian influenza A on birds and the permanently changing epizootic situation in the Balkans, Europe and Worldwide require a constant update of the National AIV Surveillance Program, promptly informing competent authorities and application of permanent biosecurity measures on farms and production units in the poultry sector in Bulgaria.

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CONFLICT OF INTEREST

There is no conflict of interest.

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