https://doi.org/10.17221/148/2019-VETMED

Analysis of a highly pathogenic avian influenza (H5N1) virus causing the first outbreak in domestic poultry in Bulgaria in January 2015

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Citation: Stoimenov G, Goujgoulova G, Hristov K (2020): Analysis of a highly pathogenic avian influenza (H5N1) virus causing the first outbreak in domestic poultry in Bulgaria in January 2015. Vet Med-Czech 65, 435–444.

Abstract: This study documents the clinical signs, necropsy findings and viral antigen distribution of the highly pathogenic avian influenza (HPAI) H5N1 virus infection in domestic poultry (a backyard farm) and the phylogenetic analysis of the virus. On January 29, 2015, an outbreak of HPAI H5N1 in domestic poultry was reported on a backyard farm in Bulgaria. Out of the twenty-two chickens with clinical signs, twenty died while the remaining two were destroyed. The morbidity was 100%, whereas the overall mortality and lethality were 90.91%. The clinical observations made were sudden death, high mortality, weakness, and recumbency. Although multisystemic lesions were observed occasionally, the main pathologic findings were observed in the nervous, circulatory, respiratory, and gastrointestinal systems. An influenza virus nucleocapsid protein was identified by an immunohistochemical analysis in all the analysed organs: brain 3/3, trachea 3/3, lung 3/3, intestine 3/3, heart 3/3, which confirmed the systemic infection. The phylogenetic analyses of the virus showed a close genetic relationship with the H5N1 viruses of Asian origin, isolated in 2012 and 2013, belonging to the clade 2.3.2.1c. The HA-gene genetically clusters with HPAI H5N1 viruses isolated from wild pelicans in Romania and Bulgaria, thereby demonstrating the link between wild and domestic birds in the epidemiology of avian influenza. The contact between the affected chickens and migrating water birds over Bulgaria's territory was suspected as a reason for the outbreak in the backyard farm. In addition, the detection of the virus in wild bird populations in Bulgaria three days earlier strongly supports the hypothesis of migrating wild birds spreading HPAI H5N1.

Keywords: histopathology; influenza virus; immunohistochemistry; phylogenetic analysis

Influenza A viruses belong to the *Orthomyxoviridae* family. Within the genus influenza virus A, many serologically different variants are classified. Up to now, sixteen haemagglutinin (HA) subtypes and nine neuraminidase (NA) subtypes have been identified in birds (Bertran et al. 2014). The two subtypes identified most recently, H17N10 and H18N11, were isolated from bats in Guatemala and Peru, respectively (Tong et al. 2012).

Some influenza A viral infections can cause significant diseases in many animal species and humans.

Their zoonotic potential has constantly been a concern to scientists and to the public's health.

https://doi.org/10.17221/148/2019-VETMED

Influenza subtypes are divided into either highly pathogenic (HPAI) or low pathogenic (LPAI). Wild birds, especially waterfowl, are known as a natural reservoir for avian influenza A viruses (AIV) and a major factor for their spreading. HPAI viruses are classified based on their ability to cause diseases in chickens, measured by the so-called intravenous pathogenic index (IVPI) above 1.2 in six-week-old chicks, which means to promote a mortality rate greater than 75% in intravenously infected 4–8-week-old chicks. Alternatively, they can be any infection with influenza A viruses of subtypes H5 or H7 with a nucleotide sequence showing the presence of multiple basic amino acids [arginine (R) and lysine (K)] at the cleavage site of haemagglutinin (OIE 2015).

In 1996, an HPAI virus of subtype H5N1 was reported for the first time in geese in Hong Kong. A year later in 1997, total of sixteen cases of infections in humans, including six deaths, were registered (Shortridge et al. 2000).

The isolated virus had the characteristic motif of basic amino acids in the cleavage site of the HA, and it was shown that the strain infecting humans was identical to that found in domestic birds (Suarez et al. 1998).

The death of a large numbers of geese in China during 1996 allowed the data about the predecessor of this virus to be collected (Tang et al. 1998). In the eighteen years between 1996 and 2014, a constant antigenic drift in the H5 gene has led HPAI H5N1 to significantly evolve, resulting in many different subtypes being generated in this time (Sonnberg et al. 2013). The A/Goose/Guangdong/96 (H5N1) strain was used for the formation of the classification of H5, and since 2008, H5 viruses are grouped into twenty genetic subtypes called clades.

Up to 2014, even more such subtypes were described, each designated by Arabic numbering (e.g., clade 1; clade 2; etc.) (WHO/OIE/FAO H5N1 Evolution Working Group 2012). Of these, the main circulating virus subtypes are from clades 1, 2.1.3, 2.2, 2.2.1, 2.3.2, 2.3.4 and 7.

The history of HPAI in Bulgaria commenced in 2006 with the occurrence of H5N1 in swans and geese (Goujgoulova and Oreshkova 2007), while H5N1 was reported in a common buzzard in 2010 (Marinova-Petkova et al. 2012). However, these cases were observed in wild birds without a mass distribution or dissemination of the disease.

An HPAI outbreak of domestic poultry in Bulgaria was reported for the first time on January 29, 2015 in a backyard farm with twenty-two laying hens, in the village Konstantinovo, the Burgas district, which is located close to the one major migratory pathways of wild birds, part of the Black Sea – Mediterranean migration route, Via Pontica. The main points where the migrating birds stop to rest at, and some of them remain and nest there, are: Pomorie Lake, Atanasovsko Lake, Mandrensko Lake and Bourgas Lake (Vaya) which are at very close distance-wise to the village Konstantinovo. Poultry farming is one of the traditional breeding industries for Bulgaria. In almost all the areas of the country there are commercial poultry sites. At the same time, there are many "backyard" farms where the level of biosecurity is low. In addition, Bulgaria's market share of fattened duck liver in Europe is over 20%, and it is well known that among the domestic duck population, a large number of LPAI viruses are circulating. The reasons for this phenomenon is the way domestic waterfowl are reared and kept in addition to the poor biosecurity measures on the farms allowing LPAIV to enter the farms through the wild birds. The combination of all this and the emergence of HPAI H5 viruses and its spread through Bulgaria by large wild bird populations via two main migratory pathways easily and efficiently enables outbreaks of HPAI. The mixing of feral and domestic waterfowl allows the development of a disease like HPAI with unpredictable economic consequences and losses.

The study of the histopathological changes in various bird species caused by the H5N1 viral infection is also essential in understanding their role in the propagation of this highly virulent virus.

MATERIAL AND METHODS

Carcasses of three domestic poultry individuals from this first outbreak underwent a necropsy in the national reference laboratory for influenza A and the Newcastle Disease part of National Diagnostic and Research Veterinary Medical Institute (NDRVMI), 190 Lomsko Shose Blvd. 1231, Sofia. Their visceral organs and brains were examined macroscopically for gross lesions. The tissues were in good *post mortem* condition and a low degree of autolysis was collected aseptically. From each selected organ, a piece was used for the virus isolation

https://doi.org/10.17221/148/2019-VETMED

(VI) and polymerase chain reaction (PCR), and another piece for the histopathology and immunohistochemistry. For the VI, a 10% suspension (w/v) of a ground sample was prepared in a Minimum Essential Media (MEM) (pH 7.2–7.4), supplemented with Streptomycin (200 mg/l), Penicillin G (2×10^6 IU/l), Nystatin dehydrate (0.5×10^6 IU/l), Polymyxin B (2×10^6 IU/l), Gentamicin sulfate (250 mg/l), and Sulfamethoxazole (200 mg/l).

Virus isolation and identification

The organs from each bird were pooled, homogenised, and the samples were centrifuged at 800 *g* for 10 min at 4 °C. An inoculation into the allantoic cavity of three 10-day-old embryonated chicken eggs (ECE) was then performed using 200 µl of the supernatant from each organ sample. The infected embryos were incubated at 36 °C for up to 96 h and checked daily for the embryo death. Subsequently, the allantoic fluids were tested for the 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 a 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 2015). The organs from each carcass were microbiologically tested to exclude a bacteriological infection as a cause of the mass mortality.

Nucleic acid detection

The supernatants of the tissue homogenates were tested using a real time reverse transcription polymerase chain reaction (rRT-PCR) by matrix AIV gene. The RNA extractions were performed with a QIAamp® Viral RNA Mini Kit (Qiagen, Venlo, Netherlands). We used a one-step rRT-PCR with AcuFlock® Influenza A Virus real-time RT-PCR Kit for the M-gene detection (the specific primers and probe included in the kit) following the manufacturer's protocol (AnDia Tec®, Kornwestheim, Germany). The M-positive samples were subtyped (H5, H7) using the RT-PCR-QIAGEN one-step RT-PCR Kit according to Slomka et al. (2007) and N1 (Slomka et al. 2012). The sequencing was performed in the Animal and Plant Health Agency, UK.

Histopathology and immunohistochemistry (IHC)

The tissue samples were immediately fixed in 10% buffered formalin for the subsequent histopathological examination. After that they were routinely dehydrated, paraffin embedded, sectioned at 5 µm and stained with haematoxylin and eosin (H&E). Duplicate sections were immunohistochemically analysed to determine the distribution of the influenza virus antigens in the individual tissues. Briefly, the sections were stained with a mouse monoclonal antibody against the influenza A virus nucleoprotein [Anti-Influenza A Virus Nucleoprotein antibody (AA5H); Abcam, Cambridge, MA, USA], followed by a biotinylated goat anti-mouse IgG secondary antibody. The bound antibodies were detected with an avidin-biotin detection system (Ventana Medical Systems, Tucson, AZ, USA). The RedMap kit (Ventana Medical Systems, Tucson, Arizona, USA) served as the substrate chromogen. The histopathological lesions were observed and documented with a Leica DM 2500 microscope equipped with a digital camera and original software.

Phylogenetic analysis

We performed a phylogenetic analysis of segment 4 (HA) of A/chicken/Bulgaria/5409/2015. The processing sequence and the preparation of the phylogenetic trees were completed with the help of the MEGA software, v7.0 (Kumar et al. 2016). Identification of the amino acid motif at the cleavage site of the HA was performed to establish the pathogenicity of strain using the program BioEdit. The alignment of the sequences was performed by the ClustalW tool in the MEGA 7.0 program. In the construction of the phylogenetic trees, the Neighbour-Joining method, (maximum composite likelihood model) was implemented (Tamura et al. 2004).

We applied the Bootstrap method (probability confidence) using 1 000 bootstrap replicates in order to confirm the topography of the branches of the phylogenetic trees (Felsenstein 1985).

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RESULTS

Epidemiological data

A poultry infection outbreak was reported on January 29, 2015 on a backyard farm in the village of Konstaninovo, in the Bourgas District. Out of the twenty-two chickens with clinical signs, twenty died while the remaining two were destroyed. The morbidity was 100%, whereas the overall mortality and lethality was 90.91%.

Two days earlier on January 27, 2015, Bulgaria reported an HPAI H5N1 outbreak in wild birds (Dalmatian pelicans) in the same district.

Two more outbreaks were reported in wild synanthropic (rock dove, black headed gull) and migratory (Dalmatian pelicans) birds on February 11, 2015 and March 26, 2015, respectively (Figure 1).

The observed and reported signs were sudden death, high mortality, weakness, and recumbency.

Gross lesions

The symptoms of the disease were observed in all of the investigated chicken. Upon the gross examination, the infected birds had characteristic pathological lesions (Figure 2A).

These included a haemorrhagic small intestine, petechial haemorrhages on the mucosa and an abnormally low content in the gizzard, and well-defined hyperaemia of the tracheal mucosa associated with petechiae.

Petechial haemorrhages were also found on the epicardial surface of the base of the heart (Figure 2B), as well as on the spleen, parietal peritoneum and intestine.

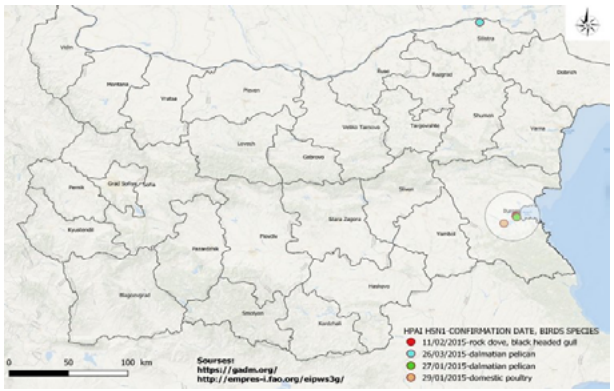

Figure 1. H5N1 highly pathogenic avian influenza events in Bulgaria 2015

Histopathological findings

The desquamation of the epithelial cells, mononuclear infiltration in the propria, hyperaemia and oedema of the tracheal mucosa with a loss of the mucosal glands were observed following the histopathological examination (Figure 3A). Congestion and a mononuclear infiltrate in the small intestine, as well as the necrosis of the lymphoid clusters in the lamina propria (Figure 3B) were present. The lung was characterised by well-expressed congestion and a pulmonary oedema

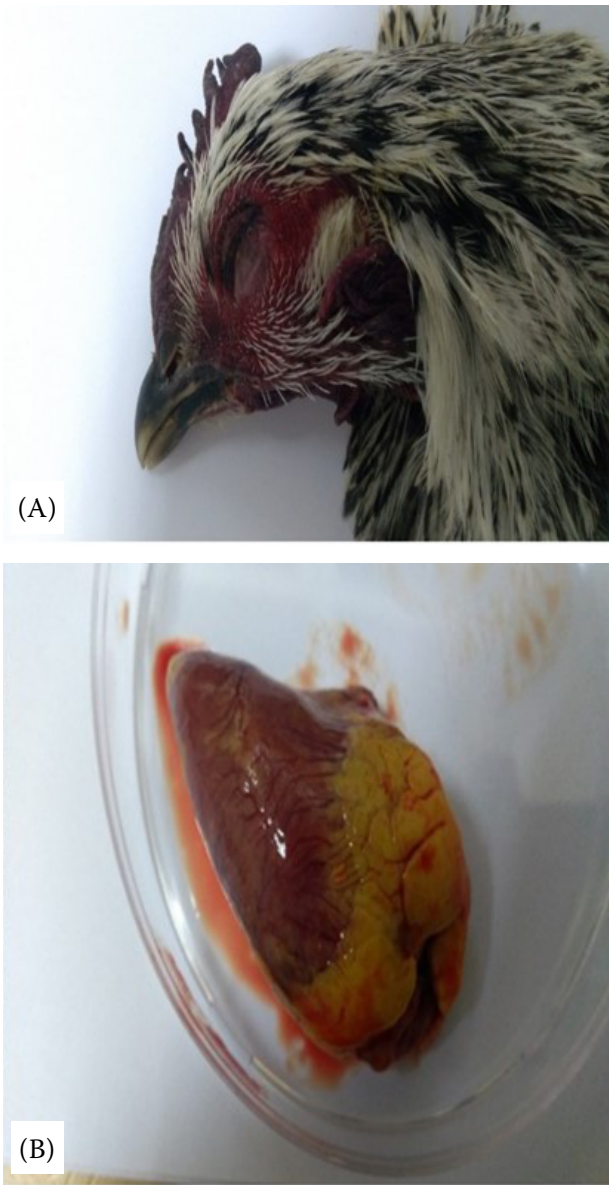

Figure 2. Gross pathologic lesions
(A) Petechial haemorrhages – epicardial surace of the base of the hearth. (B) Cyanosis (blue discoloration) of the comb and wattles

https://doi.org/10.17221/148/2019-VETMED

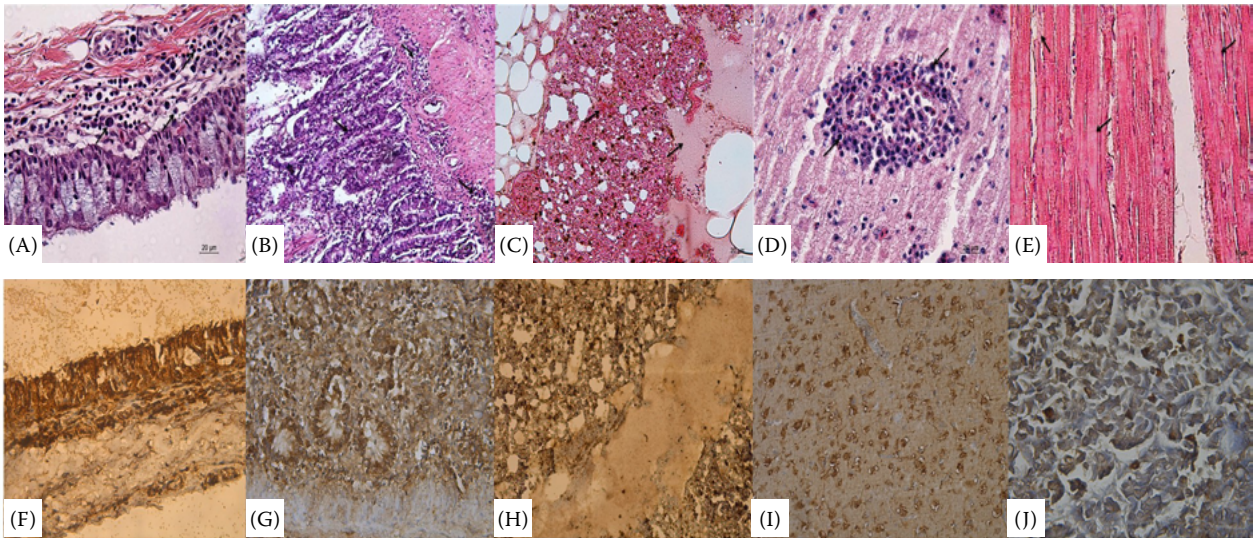

Figure 3. Histopathological and IHC changes

(A) Trachea – acute tracheitis, with epithelial desquamation, 200 ×; (B) Small intestine – necrosis of the lymphoid clusters in the lamina propria 200 ×; (C) Lung – haemorrhagic pneumonia, oedema, emphysema, and atelectasis, 100 ×; (D) Brain – perivascular and pericellular oedema, 200 ×; (E) Myocardium – interstitial oedema, 100 ×; (F) Trachea – IHC expression of the anti-influenza A virus nucleoprotein antibody, 100 ×; (G) Small intestine – IHC expression of the anti-influenza A virus nucleoprotein antibody, 100 ×; (H) Lung – IHC expression of the anti-influenza A virus nucleoprotein antibody, 200 ×; (I) Brain – IHC expression of the anti-influenza A virus nucleoprotein antibody, 200 ×; (J) Myocardium – IHC expression of the anti-influenza A virus nucleoprotein antibody, 400 ×

(Figure 3C). Microscopically in the brain, congestion with a small focal necrosis and gliosis with multifocal non-purulent encephalitis were observed (Figure 3D). A myocardial interstitial oedema and degenerative necrobiotic processes were detected (Figure 3E). The influenza virus nucleocapsid protein was identified by the immunohistological analysis in all the analysed organs:

1) tracheal epithelia 3/3 (Figure 3F),
2) intestine 3/3 (Figure 3G),
3) lung 3/3 (Figure 3H),
4) brain 3/3 (Figure 3I),
5) cardiomyocytes 3/3 (Figure 3J).

Immunoreactivity was observed in the areas with or without microscopic lesions.

Virus isolation and identification

AIV was isolated from the visceral organs of the affected birds. The allantoic fluid from the dead embryos was shown to be positive for haemagglutination activity. The following HI assays with different positive sera (as specified in the OIE 2015), established that the isolates belong to the H5 subtype.

Nucleic acid detection and sequence analysis

The PCR detection of the M-gene, followed by the specific rRT-PCR for H5N1-analysis showed that the H5N1 AIV was present in the visceral organs of all the birds examined in this study.

After the sequence analysis of the isolates, the characteristic motif of the basic amino acids in the cleavage site of the HA was found, which is typical of HPAIVs (Horimoto and Kawaoka 1994).

Phylogenetic analysis

Phylogenetic analysis of segment 4 (HA) indicated a close genetic homology with the Asian H5N1 viruses isolated in 2012 and 2013 belonging to the clade 2.3.2.1c with the Bulgarian isolate.

The nucleotide sequence showed a genetic proximity to A/Dalmatian pelican/Bulgaria/2015 (H5N1) – 99.9%, A/environment/ Huzhou/ C291-7/2013(H5) – 98.8%, A/Alberta/01/2014) – 98.6%, and A/tiger/Jiangsu/01/2013 (H5N1) 98.3% (Figures 4 and 5).

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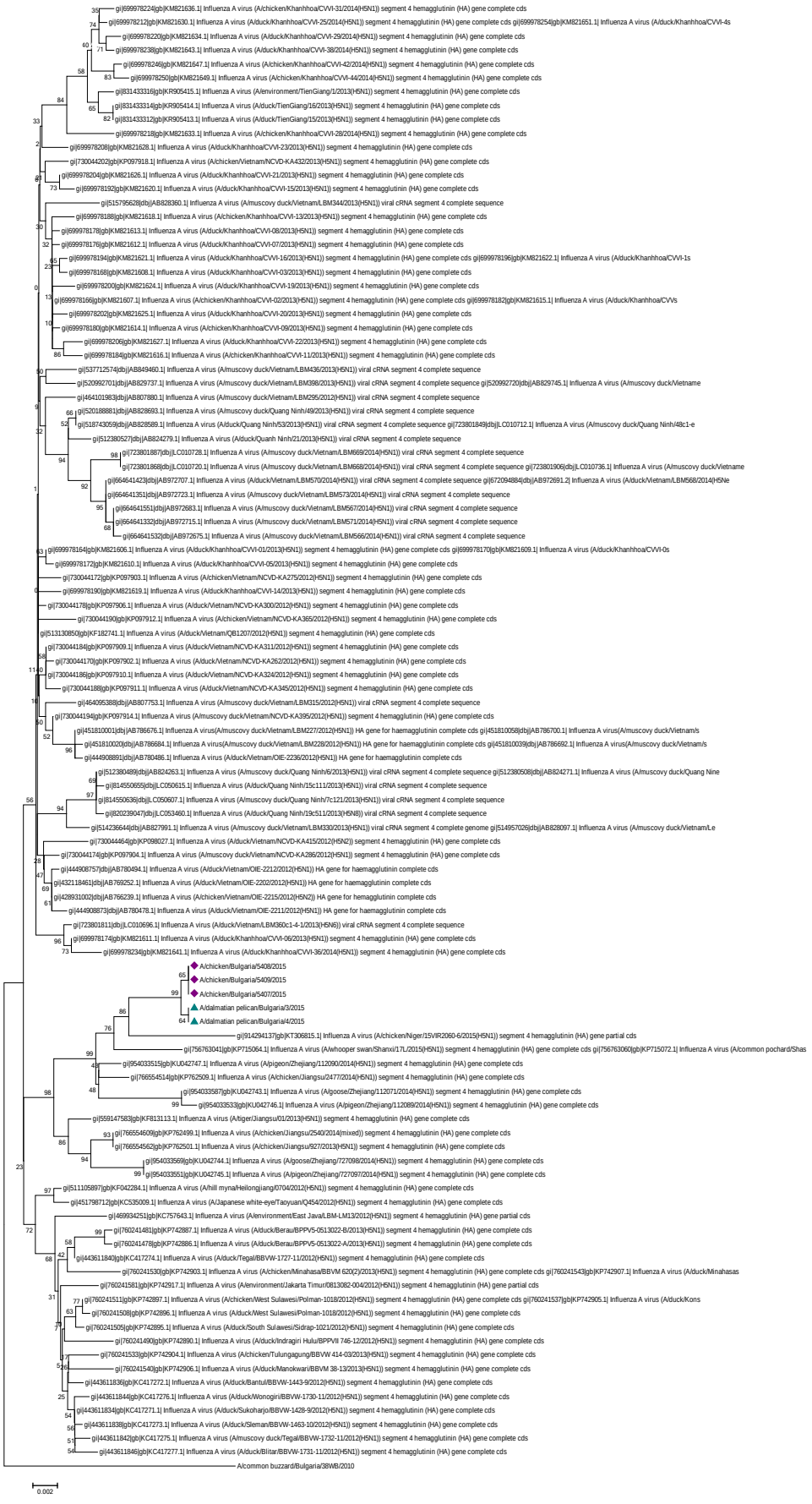

Figure 4. The phylogenetic tree of the HA-gene showing a close genetic relationship with the Vietnamese H5N1 isolated in 2012 and 2013 belonging to clade 2.3.2.1c and also with the other Bulgarian viruses (isolated in domestic poultry marked with a red rectangle and wild birds marked with a green triangle) detected in early 2015. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.220 690 35 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches. The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 106 nucleotide sequences. The codon positions included were the 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All the positions containing gaps and missing data were eliminated. There were a total of 1 640 positions in the final dataset. The evolutionary analyses were conducted in MEGA7

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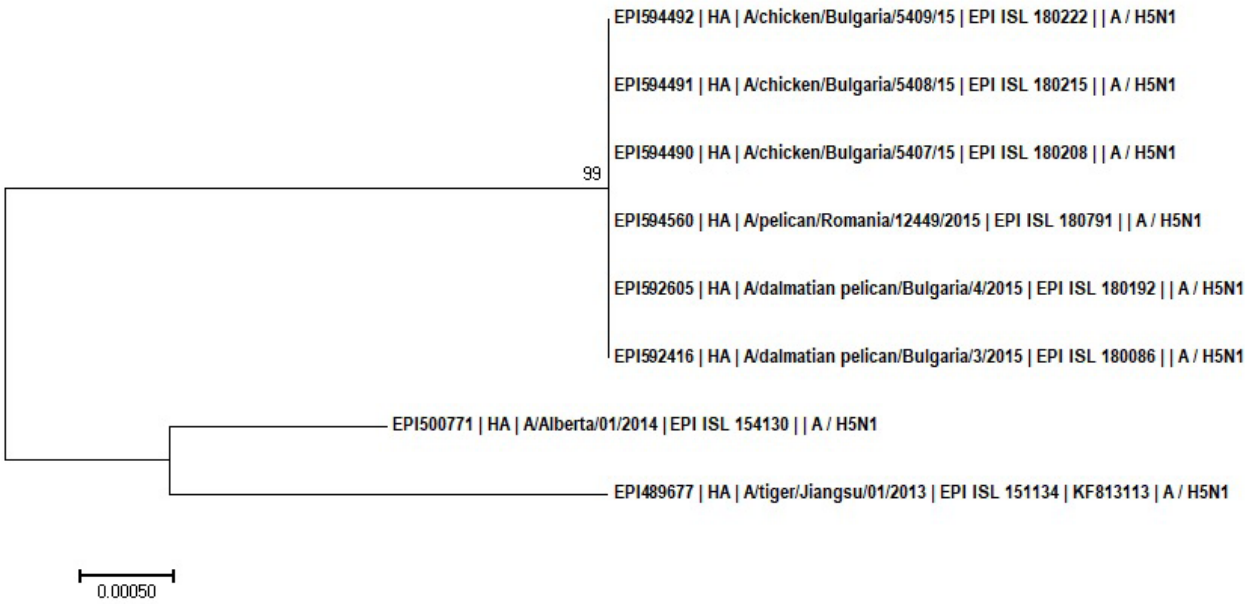

Figure 5. A closer view of the phylogenetic tree of the HA-gene showing the close genetic relationship with the H5N1 viruses isolated in 2013 and 2014 belonging to clade 2.3.2.1c and also with the Romanian and the other Bulgarian viruses detected in early 2015

DISCUSSION

HPAI viruses in chicken species are known to cause a variety of septicaemia and inflammatory-necrotic changes affecting the visceral organs and skin. The HPAI in these species is associated with high morbidity and mortality (Swayne and Suarez 2000). It is known that wild water birds play a key role in the ecology of the disease, being the main reservoir and vector of AIV (Hinshaw et al. 1980; Suss et al. 1994).

Lesions affecting the heart were a recurrent histopathological finding in our study. Severe manifestations of oedema in the myocardium may be associated with endothelial damage and the increased permeability of the coronary vessels in this case. This is a result of a fatal systemic disease known to be caused by HPAI H5 or H7 viruses in gallinaceous birds (Swayne 2007). The intensive oedematous lesions could be a direct cause of atrophic and destructive myofibrillar alterations. The high-level extravasation and large fluid collection in the pericardial sac must also be associated with the possibility that the coronary endothelium is a target for HPAI viruses. The IHC examinations validated the lesions in the myocardium as infarction and found that the multifocal necrosis in the myocardium is associated primarily to virus replication and not to vascular changes.

Evidence of marked congestion, oedemas, and haemorrhages was shown in the lungs. Congestion and pulmonary oedemas are non-specific changes most likely related to heart failure due to necrotising myocarditis. The demonstration of the AIV antigen in the pulmonary endothelium by IHC allows one to conclude that these lesions are specific for the virus infection. HPAI viruses can cause damage in cells and lead to cell death through necrosis or apoptosis. Necrosis is associated with the direct virus replication in cells due to the high accumulation of the viral nucleoprotein in the cytoplasm and nucleus of the infected cells (Swayne 2007). Neurological clinical signs and observed congestion with small areas of necrosis and gliosis with multifocal encephalitis confirm the neurotropism of the virus. The number of domestic chickens with encephalitis, in association with high levels of the viral nucleoprotein as detected by IHC, indicate the highly neurotropic nature of the virus, as shown by previous studies (Brown et al. 2006; Pasick et al. 2007).

Bulgaria has a peculiar geographic location in Europe and the Balkans. It is known that its territory is crossed over by two major migratory pathways of wild birds, part of the Black Sea – Mediterranean migration route. The main points where the migrating birds stop to rest at, and some of them remain and nest there, are: Pomorie Lake, Atanasovsko Lake,

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Mandrensko Lake and Bourgas Lake (Vaya). The history of HPAI in Bulgaria until 2015, commenced in 2006 with the occurrence of H5N1 in swans and geese (Goujgoulova and Oreshkova 2007), and continued in 2010, where HPAI H5N1 was reported in the common buzzard (Marinova-Petkova et al. 2012). Cases of an HPAI (H5N1) infection in 2015 had been reported both in wild birds (3 outbreaks) and in a backyard farm (1 outbreak). Given the complicated epidemic situation in Asia and Europe, and the passage through Bulgaria of two major migratory routes from Asia to Africa, this was an expected scenario. The introduction of the infectious agent in the backyard farm is most likely due to the wild migratory birds. At the beginning of 2015, the HPAI H5N1 virus was found in domestic chickens (*Gallus Gallus domesticus*) housed in a backyard farm in the Burgas region. The proximity of the farm to Pomorie Lake – 20 km, Atanasovsko Lake – 15 km, Mandrensko Lake – 2 km and Bourgas Lake (Vaya) – 12 km, where many wild water birds spend the winter, may play a key role in the introduction of the infection in the farm. In addition, the detection of the virus in wild bird populations, 3 days earlier and the phylogenetic analysis of the viruses strongly support the association between the cases in the wild birds and the outbreak in the backyard farm. The phylogenetic analysis of segment 4 (HA-gene) of A/chicken/Bulgaria/H5N1/2015 confirms the Asian origin of the virus, showing the genetic proximity to A/environment/ Huzhou/C291-7/ 2013(H5) – 98.8%, A/Alberta/01/2014 (H5N1) – 98.6%, and A/tiger/Jiangsu/01/2013 (H5N1) 98.3%. The results of the phylogenetic analysis and comparison of the HA-gene of the isolates from Bulgaria and Romania support the hypothesis for the spread of the virus in both countries through these migratory patterns. After its emergence in Europe at the beginning of 2015, HPAI H5N1 clade 2.3.2.1c was detected in Africa (Ivory Coast, Burkina Faso, Niger and Ghana). The phylogenetic analysis of the African isolates found that the virus may have entered Africa via two different routes. They formed two branches, one of them being phylogenetically closer to those viruses isolated in Bulgaria and Romania – 99.3% and 99.65%, respectively (Tassoni et al. 2016). The results suggest that the virus may have entered Africa in different ways, one of which is through migratory patterns.

Influenza A infections pose a significant threat to the animals and the public's health. The zoonotic potential of HPAI H5N1 has always inspired a great interest among scientists and provided challenges to public health authorities. The detection of the HPAI H5N1 clades 2.3.2.1c in Southeast Asia and Bulgaria (in wild birds) for the second time since 2010 (Marinova-Petkova et al. 2012) shows their potential for a trans-continental distribution. The combination of low biosecurity levels in "backyard" farms, the large numbers of farms for fattening ducks (it is well known that among mule ducks, a large number of low pathogenic influenza A viruses are circulating, which is related to the way in which these birds are kept and the biosecurity measures taken) and the passage through Bulgaria of large populations of wild birds through two main migratory pathways continues to favour future outbreaks of avian influenza A. Strict compliance with biosecurity measures in industrial poultry farms and increased vigilance of veterinary services and farmers in backyard farms and in farms with fattening ducks are crucial for the effective control of the disease.

This study documents the clinical signs, the pathological findings and the viral antigen distribution of the natural HPAI H5N1 infection in domestic poultry (backyard farms) as well as the phylogenetic analysis of the virus. Variable necrosis was observed in the brain, trachea, heart, and small intestine of the examined birds. A viral antigen was commonly found in the brain, heart, lung, intestine and trachea of the said birds. The phylogenetic analyses of the A/chicken/Bulgaria/2015 (H5N1) showed the closest genetic relationship with the Vietnamese H5N1 virus isolated in 2012 and 2013 belonging to clade 2.3.2.1c and also with the Romanian and the other Bulgarian viruses detected in early 2015.

These results support the hypothesis that the virus spread throughout Bulgaria and Romania through the migratory patterns of wild birds. The proximity of the backyard farm to many wild waterfowl bird' habitats, where many of them spend the winter, plays a key role in the introduction of the infection to the farms.

Acknowledgement

The authors would like to thank Ian Brown, Steve Essen, James Seekings, Reneta Petrova, Julian Ananiev and John Bianco for their valuable help.

https://doi.org/10.17221/148/2019-VETMED

Conflict of interest

The authors declare no conflict of interest.

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Received: October 18, 2019
Accepted: August 30, 2020