



## HIGHLY PATHOGENIC AVIAN INFLUENZA IN BULGARIA – A REVIEW

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### Summary

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The aim of this review was to summarise the information about the cases of highly pathogenic avian influenza in Bulgaria during the last two decades. According to the EMPRES-I FAO data, from January 2006 to the end of 2019, 141 HPAI outbreaks have been registered in Bulgaria, with two identified virus serotypes (H5N1 and H5N8). The H5N1 outbreaks were reported in 2006, 2010 and 2015. Almost all H5N1 cases were observed in wild birds, with only one outbreak in domestic chickens in a backyard farm in 2015. From 2016 to the end of 2019, 132 outbreaks of HPAI identified as H5N8 were recorded: sixteen in wild birds and the other 116 – in domestic poultry. Large farms with fattening ducks used for foie gras production in four administrative districts (Plovdiv, Haskovo, Stara Zagora, Dobrich) were mainly affected.

**Key words:** foie gras, influenza, domestic poultry, wild birds

### INTRODUCTION

Influenza A viral infections are hazardous important diseases in many animal species and humans. Their zoonotic potential has always sparked a keen interest in scientists and instil fear in the population. It is considered that wild birds and especially waterfowl are a natural reservoir for the influenza A viruses and a significant factor for their spreading (Olsen *et al.*, 2006). They are also the primary source of all avian influenza virus (AIV) subtypes. However, the joint effect of interactions between these different host populations and viruses has still received inadequate

attention. The presence of strains of highly pathogenic influenza A (HPAI) in wild birds, which not always leads to a lethal infection in them, mediates the transfer of these viruses at a great distance through migration. This reinforces the need for a much detailed study of the natural evolutionary history of AIV around the world.

Avian influenza, commonly known as fowl plague, is caused by viruses with segmented, negative-sense, single-stranded RNA genome belonging to the influenza virus A genus of the *Orthomyxoviri-*

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*dae* family. AIVs are divided into subtypes based on the antigenic surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). To date, 16 HA (H1 to H16) and 9 NA (N1 to N9) AIV subtypes have been identified in birds (Bertran *et al.*, 2014). AIVs are classified as highly pathogenic (HP) for poultry when the intravenous pathogenicity index (IVPI) in six-week-old chickens is either higher than 1.2 or causes at least 75% mortality in four-to-eight-week-old chickens infected intravenously. The same classification applies when the characteristic motif of basic amino acids in the cleavage site of HA is identified after sequence analysis. Low pathogenicity avian influenza viruses are those (including H5 and H7 subtypes) that do not meet the above criteria for highly pathogenic avian influenza (OIE, 2019).

Three sets of factors influence the emergence and development of the infectious process: characteristics of the infectious agent, the properties and condition of the macro-organism and state of the environment, in which the interaction takes place. This simple concept is essential not only for studying the epidemiology of the disease in wild birds but also for establishing the potential impacts associated with the introduction of HPAI viruses in wild and domestic bird populations.

The proximity of wild birds to industrial poultry farms poses a potential threat for development of a disease with unpredictable economic consequences and losses. Bulgaria has a particular geographic location in Europe and the Balkans. Two major migratory paths of wild birds cross over its territory, part of the Black Sea-Mediterranean migration route: Via Pontica and Via Aristoteles. The former that is more critical is one of the main

migration routes for birds from Western Siberia (Asia) and North and Eastern Europe to Africa. It passes through the territory of Bulgaria above Strandzha Mountains and along the coast of the Black Sea and the wetlands connected with it. The main sites 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). Another major stop for the migrating birds is the area of Bolata – north of Cape Kaliakra. Apart from Via Pontica, another migratory path of birds passes through Bulgaria (Via Aristoteles) – through the Struma River Valley, the Sofia Field and the Iskar Gorge. Birds migrate in the autumn to the south and return on the following spring.

Poultry farming is one of the traditional breeding industries for Bulgaria. Industrial poultry operations are present in almost all areas of the country. 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 known that a large number of low pathogenic influenza A viruses circulates among the ducks used for foie gras production, related to the way these birds are kept and the biosecurity measures on these farms. This poses a serious danger for introducing viruses from wild birds and the epizootic development. The combination of all listed circumstances and the passage through Bulgaria of large wild bird populations through the two main migratory pathways creates potential conditions for avian influenza outbreaks. Strict compliance with biosecurity measures in industrial poultry farms and increased vigilance of veterinary services and farmers in backyard farms are neces-

sary requirements for effective control of the disease.

#### HIGHLY PATHOGENIC AVIAN INFLUENZA SUBTYPE H5N1

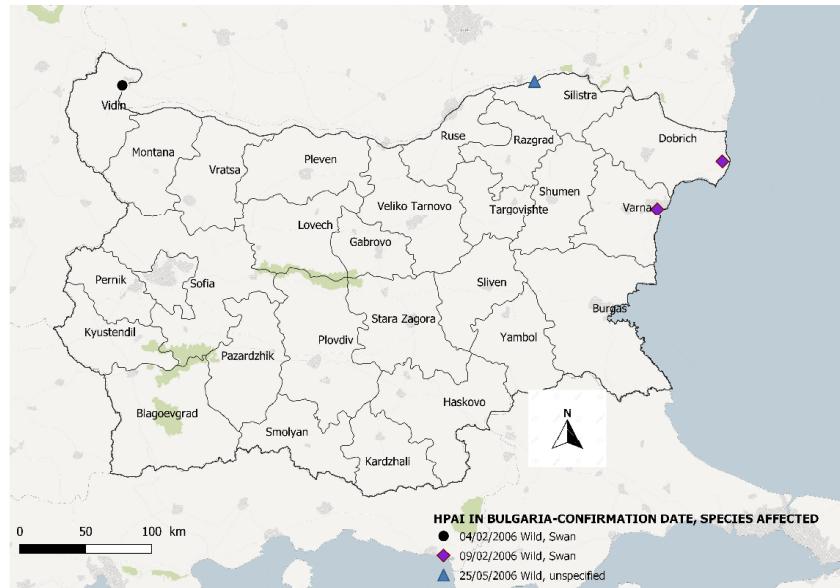
Serious talks about avian influenza in Bulgaria started in October 2005, after the incidence of severe outbreaks, caused by H5N1 in neighbour countries Turkey and Romania. Long before 2005, Bulgarian scientists have studied the influenza viruses of type A, including avian influenza. Galabov *et al.* (1994) researched the effect of various antiviral drugs that may potentially combat influenza in people.

In 2006, HPAI H5N1 was isolated for the first time from birds (Fig. 1). After sequencing of the haemagglutinin gene of the four H5N1 isolates it was found that they contained the same motif of basic amino acids in the cleavage site, namely

PQGERRRKRR \* GLF, i.e. they were HPAI strains (Goujgoulova *et al.*, 2007).

The phylogenetic analysis inferred a close relationship with H5N1 strains which were isolated during epizootics in Europe, Middle East and Africa. These viruses belonged to genotype "Z" of the highly pathogenic influenza subtype H5N1 or the so-called "Asian lineage HPAI A (H5N1)", clade 2.2 (Goujgoulova, 2010).

In the spring of 2010, HPAI H5N1 from a different clade – 2.3.2.1c – was isolated for the first time in Bulgaria from a common buzzard (*Buteo buteo*) (Marianova-Petkova *et al.*, 2012). The HA gene was closely related to A/great crested grebe/Qinghai/1/2009 and A/grebe/Tyva/3/2009, with an overlap of 100 and 98%, respectively, and a maximum identity of 99% for both viruses. Identification of the molecular characteristics of the virus showed multiple basic amino acids in the



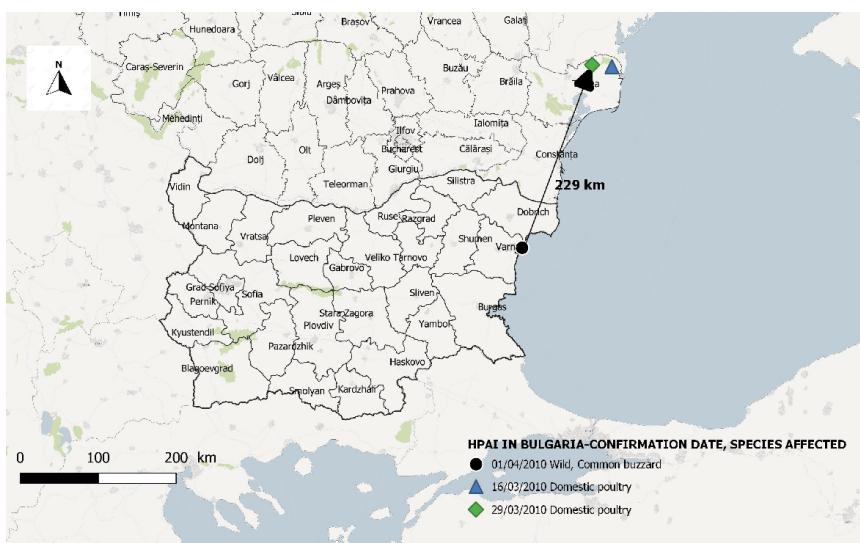
**Fig. 1.** Highly pathogenic avian influenza H5N1 outbreaks in Bulgaria, 2006  
<https://www.qgis.org/en/site/getinvolved/styleguide.html>;  
<http://empres-i.fao.org/eipws3g/>; <https://gadm.org/>).

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cleavage site of their HA protein – PQRERRRKRGFL, which is highly characteristic of HPAI (Claas *et al.*, 1998), as well as K329 deletion (at H5 numbering) at the same site. The location at which HA1 binds to the receptors of the host cells contains amino acid residues Glu 186 (E), Gln 222 (Q) and Gly 224 (G), which bind predominantly to  $\alpha$ -2,3-NeuAcGal receptors (Ha *et al.*, 2001). The H5N1 virus, isolated from a common buzzard, possessed a molecular marker characteristic for high pathogenicity in hens but had no mutations allowing it to bind to human-type receptors on the respiratory epithelium (Marinova-Petkova *et al.*, 2012). The phylogenetic analysis showed that from an evolutionary aspect, HA from A/Common buzzard/Bulgaria/38WB/2010 was most closely related to H5N1 viruses, isolated from whooper swans in Mongolia in 2010 and 2009, as well as to a virus, isolated from a Great crested grebe in 2009 in the Russian Republic of Tuva, bordering with Mongolia.

Clade 2.3.2.1 viruses, among which the Bulgarian virus, were isolated only from wild migratory waterfowl and coastal birds in the period 2009–2010. Probably, the maintenance of this group of viruses in wild bird populations is responsible for its wide geographical spread in Asia, namely in China, Hong Kong, Korea, Vietnam, Laos, Bangladesh, Nepal, Mongolia, Tuva (WHO, 2012).

The question though is how did the common buzzard in Bulgaria get infected? It is a bird of prey from the family *Accipitridae*. Cases of a deadly infection with H5N1 influenza viruses in birds of prey were reported (Magnino *et al.*, 2000; Manvell *et al.*, 2000; Van Borm *et al.*, 2005). Although not a waterfowl, being a predator who often feeds on carrion, a common buzzard is exposed to the risk of contracting HPAI through direct contact and via the alimentary tract upon attacking smaller dead or sick birds. Common buzzards (*Buteo buteo*) are considered to not migrate over long distances. Yet, research



**Fig. 2.** Highly pathogenic avian influenza H5N1 events in Bulgaria, 2010 and possible scenario for transmission patterns (<https://www.qgis.org/en/site/getinvolved/styleguide.html>; <http://empres-i.fao.org/eipws3g/>; <https://gadm.org/>).

conducted in Bulgaria in the period from 1979 to 2005, showed that a significantly high number of common buzzards (up to 42,100 birds) follow the Via Pontica migration route in spring and autumn (Kostadinova *et al.*, 2007) suggesting that these birds are possible carriers of pathogens over long distances. A possible scenario is that the common buzzard, at the change in weather and in search of food, had migrated for a while to Romania, where it had attacked an infected bird, either a waterfowl bird or a hen (Fig. 2). After getting infected, it had probably returned to Bulgaria, where it was soon found dead (this is explained by the absence of any lesions in the internal organs, i. e. death was very fast). The similarity between the Bulgarian and Romanian isolates in phylogenetic terms helps to clarify the picture of the spread of the virus in both countries.

In January 2015, after H5N8 foci emerged throughout Europe, H5N2 outbreaks in America of H5N2, and H5N1 clade 2.2.1.2 in the Middle East, H5N1 clade 2.3.2.1.c was once again isolated in Bulgaria. The initial outbreak was in Poda Protected Area, Burgas region, (GPS coordinates: 42.449780; 27.464885). The affected species was a Dalmatian pelican (*Pelecanus crispus*) – case fatality rate 100%. Six days later, on 30 January 2015, a secondary outbreak of the infection was reported – this time in domestic poultry, at a backyard farm in Burgas region. Twenty chickens out of the 22 with clinical signs have died, the other 2 were destroyed (morbidity 100%, overall mortality 90.91%, lethality 90.91%). At necropsy, characteristic pathological signs of HPAI (petechial haemorrhages on the internal organs and on the inside of the abdominal cavity, dark blue wattle and comb) were observed. The next outbreak of the dis-

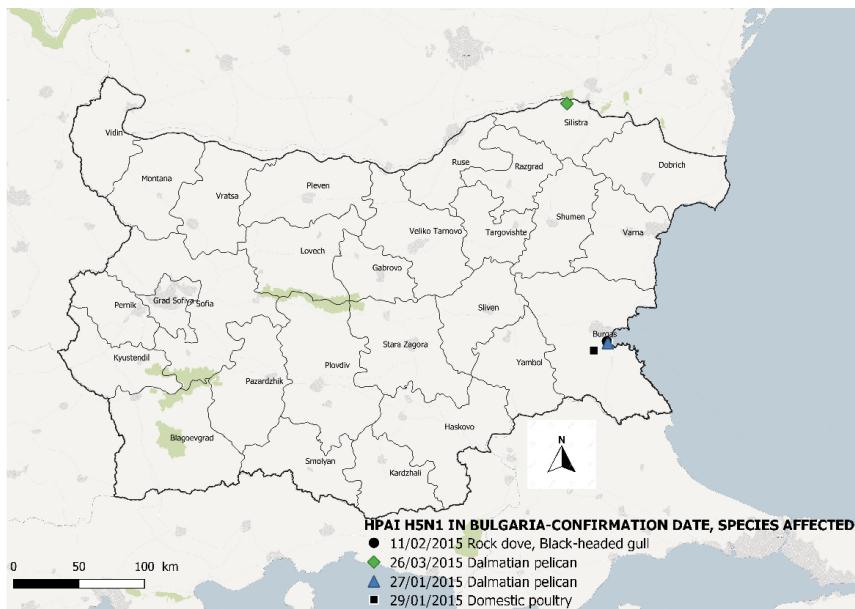
ease followed after only a few days. On 4 February 2015 highly pathogenic influenza was proved in two synanthropic birds – Black-headed gull (*Larus ridibundus*) and Rock dove (*Columba livia*), again in Burgas. The lethality was also 100%.

After a month-long pause, the fourth outbreak took place in Srebarna biosphere reserve on 22 March 2015, where Dalmatian pelicans (*Pelecanus crispus*) encountered the virus for a second time (Stoimenov *et al.*, 2018a,b). In an emergency action, the reserve staff identified 29 dead pelicans. Laboratory tests confirmed later the presence of highly pathogenic avian influenza subtype H5N1. The corpses were visibly cachectic, in poor state, their weight was barely 5 kg (normal 9–9.5 kg). At that moment there were 65 pairs of Dalmatian pelicans.

The outbreaks of infection in the country for 2015 were 4 (Fig. 3), registered from January to March. Afterwards no new disease events have occurred. The main species affected was the Dalmatian pelican, a migratory bird directly associated to aquatic habitats. Cases of HPAI in both synanthropic birds, one of which was not inherent to aquatic habitats, should be emphasised.

#### HIGHLY PATHOGENIC AVIAN INFLUENZA SUBTYPE H5N8

H5N8 Buan-like sublineages, or Group A, are viruses encountered and detected for the first time in Europe and some Asian countries in 2014. Buan-like H5N8 precursor viruses were believed to have been initially detected in eastern China before their intercontinental spread (Zhao *et al.*, 2013; Lee *et al.*, 2016; Kim *et al.*, 2017). Although outbreaks did not persist for a long period, this was the first incursion of clade 2.3.4.4 of Gs/GD lineage into



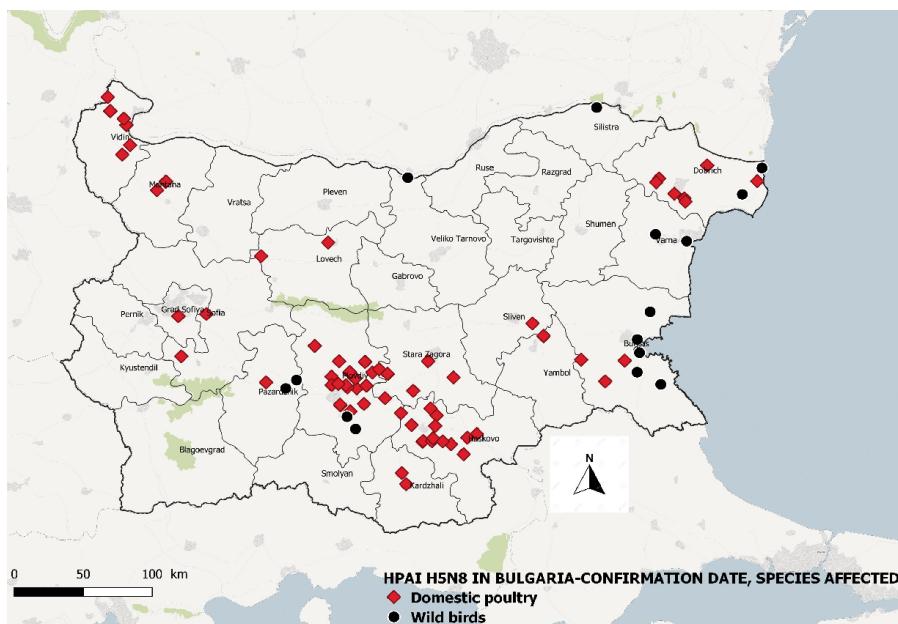
**Fig. 3.** Outbreaks of highly pathogenic avian influenza H5N1 during 2015 in Bulgaria  
<https://www.qgis.org/en/site/getinvolved/styleguide.html>;  
<http://empres-i.fao.org/eipw3g/>; <https://gadm.org/>).

Europe (Globig *et al.*, 2017). In 2016, the second wave of H5N8 was detected. This group of H5N8 was then called “Gochang-like” viruses. This novel H5N8 reassortant was detected in a wild bird in Qinghai, China in May 2016, before it persisted in intercontinental spread (El-Shesheny *et al.*, 2017; Lee *et al.*, 2017; Li *et al.*, 2017). Furthermore, this H5N8 HPAI virus group, which had also been detected in the Tuva Republic, Russia, differed from the H5N8 group, which persisted in Europe in 2014 (Lee *et al.*, 2016).

In Bulgaria, H5N8 was first recorded in 2016 (OIE, 2016) and until the end of 2019, according to FAO data, 132 epizootic outbreaks were documented. In 2016, 21 outbreaks of HPAI H5N8 have been announced, in 2017 the outbreaks were 73, in 2018 – 33, in 2019, 5 outbreaks were recorded (Fig. 4). A more detailed

review of the prevalence of the disease by years is needed. In 2016, twenty-one outbreaks were registered in seven administrative regions (Plovdiv, Pazardzhik, Sofia, Stara Zagora, Kardzali, Montana, Sofia-city), with most of the outbreaks in the Plovdiv Region – 12, all in ducks used for foie gras production, followed by Stara Zagora – 3, again in ducks used for foie gras production. A single outbreak in wild birds was reported, in a common buzzard (*Buteo buteo*). All outbreaks were declared in December 2016.

In 2017, 14 administrative regions were affected (Burgas, Varna, Dobrich, Montana, Vidin, Veliko Tarnovo, Pazardzhik, Yambol, Sliven, Haskovo, Stara Zagora, Plovdiv, Sofia), with 15 of outbreaks in wild birds. One wildlife farm cultivating common pheasants (*Phasianus colchicus*) for hunting in the village of Trunkovo, Yambol district was also af-



**Fig. 4.** The registered outbreaks of HPAI in Bulgaria for the period from 2015 to 2019, [en/site/getinvolved/styleguide.html](http://en/site/getinvolved/styleguide.html); [http://empres-i.fao.org/eipws3g/](http://http://empres-i.fao.org/eipws3g/); <https://gadm.org/>.

fected (Stoimenov *et al.*, 2019). All cases were reported in the first quarter of the year from 6 January to 14 March 2017. The regions, where wild bird outbreaks were declared, were Varna, Burgas, Plovdiv, Sofia-city, Dobrich, Veliko Tarnovo, Pazardzhik. The remaining 58 outbreaks, involving domestic poultry, were registered in Burgas, Dobrich, Montana, Vidin, Pazardzhik, Kardzhali, Yambol, Sliven, Haskovo, Stara Zagora, Plovdiv, and Sofia regions. The outbreaks of the disease in 2018 fell by more than 50% – there were 33 registered in 6 regions of the country (Plovdiv, Haskovo, Vidin, Yambol, Stara Zagora, Dobrich). Interestingly, in that year only cases in domestic poultry were reported, predominantly in ducks used for foie gras production.

In 2019, 5 outbreaks were registered in 2 administrative regions in the country (Plovdiv and Lovech), again only in domestic poultry.

Viewed by regions, the Plovdiv region was the most affected during the H5N8 epizootic with 48 registered disease events during the entire surveillance period, with only 2 occurring in wild birds. The first registered outbreak in the region was on 23 December 2016, as other outbreaks are still announced in 2019, the last ones being registered in Krumovo and Asenovgrad on 05 April 2019. The next most affected region was Haskovo with 20 outbreaks registered – all in domestic poultry, most of them in ducks used for foie gras production. The first outbreak in the region was announced on 11 January 2018, and the last was on 13 November 2018. In 2019, HPAI outbreaks in this region were not declared. The next region is Stara Zagora with 18 epizootic outbreaks (EO), none of which in wild birds. For the first time during the surveillance period, infection by HPAI (H5N8) was reported on 30 December 2016 in the vil-

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lage of Granit, whereas the last reported case was from 26 February 2018. In the Dobrich region, 11 EO were ascertained from 16 January 2017 to 17 August 2018, with only 3 being among wild birds registered in early 2017 and the remaining 8 – among domestic poultry.

A review of the epidemiological situation in the administrative region of Plovdiv and the time range in which the outbreaks occurred shows that the first cases were among domestic poultry on 28 December 2016 in the villages of Padarsko and Manolsko Konare. Then, in February and May 2017 two cases were detected among wild birds in the region of Asenovgrad. The infection in the region continued to develop during 2019.

The last case of HPAI (H5N8) virus among wild birds in the territory of Bulgaria and Europe was on 9 May 2017. In 2018, infection outbreaks significantly decreased compared to those in 2017, 33 and 73, respectively, showing a reduction of more than 50%, which has a logical explanation. Viral infection with HPAI H5N8 does not persist among wild bird populations in Bulgaria or Europe. However, it is alarming that the disease continued to develop in 2018 with 33 EOs and in 2019 with 5 EOs until the end of May.

The spread of HPAI in Haskovo and Stara Zagora administrative regions is not surprising due to the population density of the mulard ducks and their geographical proximity, which, combined with gaps in biosecurity programmes of the farms, favours the development of the infection.

In Dobrich Region, a purely epidemiological pattern was observed throughout the course of the epizootic. The first reported outbreaks were among wild birds in Kavarna and Durankulak, in January 2017, and afterwards among domestic

poultry, with the last EO being registered in August 2018.

The laboratory of the Animal and Plant Health Agency, UK was provided with 25 viruses isolated in Bulgaria from October 2017 to November 2018 to perform a complete sequence analysis to determine the genetic diversity of the viruses; likely origin; molecular epidemiology for establishing the relationship/correlation of individual outbreaks (if any) by time and region to obtain information helping surveillance and disease control strategies. The report of the Animal and Plant Health Agency, UK (Brown *et al.*, 2019) on outbreaks in Bulgaria in 2018 concluded that The phylogenetic analysis of isolated viruses in Europe in 2018 revealed that wild birds were not the main source of H5N8 infection in Bulgaria, as there was no prolonged circulation of the virus in Europe throughout the year. The last outbreak reported in wild birds, dates from the beginning of 2017 in a common buzzard – (*Buteo buteo*) that was found dead by passive surveillance in Bulgaria. Outbreaks have been registered in the country from late 2017 to late 2018 mainly in three regions – Dobrich, Yambo and Plovdiv.

### H5N8 HPAI IN BULGARIA IN 2017–2018: EPIDEMIOLOGICAL AND GENETIC ANALYSIS

#### *Summary*

Significant episodes of H5N8 HPAI outbreaks in multiple bird farms with wide geographical spread.

- H5N8 HPAI outbreaks have been ascertained from October 2017 with continued detection until November 2018.

- Three primary introductions of the infection have been ascertained using viral genetic information.
- There is no information on the spread or new introduction of the infection through wild birds.
- Epidemiological clusters of the infection were formed, linked to the geographical areas of production of the birds.
- Transmission via risky routes related to the movement of live domestic poultry and/or human-related mechanisms, i.e., people, vehicles, is likely, especially in conditions with high stocking density of birds.
- The high density of farms and the density of the duck population in the districts of Plovdiv, Haskovo, and Stara Zagora in Central Bulgaria is a key factor in maintaining the virus and expanding its genetic diversity.
- Parallels with France 2015, 2016/17 and Hungary/Bulgaria 2016/17. Once infection in the mulard ducks production sector has been identified, the specific risk is the high transmission rate to neighbouring farms, including other manufacturing sectors.

#### *Epidemiological data*

- From 17 October 2017 to the end of 2018 Bulgaria announces 32 outbreaks of HPAI H5N8 among domestic birds.
- No cases among wild birds have been reported during this period.
- The outbreaks are grouped near Plovdiv and Haskovo in Central Bulgaria, Yambol in Eastern Bulgaria and Dobrich in Northeast Bulgaria.
- A great number of bird species were affected. There is no specific predisposition to infection. Backyard farms and commercial production farms were affected.

- The morbidity among avian birds is typical of HPAI with sufficient mortality to sharpen the attention of veterinary authorities. Mortality in Anseriformes is variable, with a number of early events showing mortality, but later ones showing little or no mortality.

#### *Genetic analysis*

From the 32 reported outbreaks starting from October 2017, in Animal and Plant Health Agency, UK viruses were obtained in collaboration with NDRVMI, Sofia, from 25 outbreaks. All viruses were sequenced (HA gene of all and, where possible, complete genomes). Three clusters were identified in the HA gene, which is the primary target of the immune response (and therefore subject to the greatest variation in population infection).

- Cluster 1 contained a single strain (sample was taken on 17 October 2017 from a duck), as the closest genetic link was from viruses from wild birds in Hungary during the former year. Since October 2017 there were no other viruses found in this cluster.
- Cluster 2 was the largest cluster by number of isolates and these viruses circulated from November 2017 to October 2018. The viruses from cluster 2 were from outbreaks in several locations in Central Bulgaria, including Haskovo, Plovdiv and Yambol in chicken, duck and partridge.
- Cluster 3 contained chicken strains from Dobrich. Representatives were barely found in March, June and July 2018.

In a phylogenetic analysis of the entire genome, three different gene relatives can be identified, reflecting the above patterns of HA. However, within the cluster 2 viruses, there was additional diversity in the

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matrix protein (M) gene through reassortment. The cluster 3 viruses were genetically similar to the cluster 2 viruses only in the NS, PA, PB1, and PB2 genes. This implied three separate initial introductions/variants, followed by the additional diversity generated in cluster 2 viruses by reassorting the MP gene. Given that cluster 2 viruses circulate in a number of domestic species, including ducks, and that these duck viruses are the ancestors of a number of 'phylogenetic roots', it is likely that prolonged circulation and transmission within the domestic ducks results in additional genetic variety. This finding will be supported by serologically positive evidence from existing duck surveillance (Brown *et al.*, 2019).

In ducks, most strains of avian influenza virus replicate in the lungs and in the epithelial cells of the gastrointestinal tract of the host, as the infected birds usually show no clinical signs of the disease (Webster *et al.*, 1978). In an experimental and natural infection of ducks with LPAI viruses, isolation of the virus from the trachea, lungs, blood, kidney, liver, spleen and duodenum of infected ducks was unsuccessful, whereas high titer virus was isolated from the caecum, rectum and cloacal bursa (Webster *et al.*, 1978; Daoust *et al.*, 2011). This explains the lack of systematic infection in LPAI, compared to the pathogenesis of HPAI, in which replications of the virus occur in all organs and systems. On the other hand, recent scientific discoveries suggest a natural resistance of ducks to the AI viruses, associated to the so-called RIG-I viral sensor detected in them but missing in avian birds (Barber *et al.*, 2010). Ducks, so-called the "Trojan horses" of H5N1 influenza, can evidently host two or more influenza A viruses at the same time (Sharp *et al.*, 1997), creating perfect conditions

for reassortment (Hinshaw *et al.*, 1980). In this way, they participate in the creation of particularly dangerous viruses for the domestic poultry, which exhibit little pathogenicity to the ducks themselves. Domestic waterfowl play also a significant role in the spread and inability to eradicate the influenza A viruses as the wild birds. The viruses of AI subtype H5N8, that have been found in the country could have spread in farms through either wild migratory birds or import of ducks. It is highly possible that AI viruses, irrespective of their origin, upon entry into farms, survive soon enough in faeces, litter and water. After that, through inventory, staff movement, poor animal hygiene and the constant overlap of different age groups of breeding birds, AI viruses are transmitted from one bird to another and even spread between farms.

## CONCLUSION

For the last two decades, only 9 outbreaks of H5N1 have been recorded in Bulgaria. However, these cases were observed in wild birds, with only one outbreak in domestic chickens occurring in a backyard farm and no ensuing mass distribution or dissemination of the disease. The situation with H5N8 is completely different with many outbreaks, mainly in duck used for foie gras production. If we have to make a hypothesis for the development of the disease based on the data available for the origin of the disease in Bulgaria by years, then most likely in 2016 and 2017 the disease has been introduced into the country through migratory wild birds, as viruses of this subtype have been reported to persist in wild bird populations both in Bulgaria and Europe. The outbreaks in 2018 are most likely related to the survival of the virus in litter and environment

for a long enough time. Through inventory, staff movement, poor animal conditions and the constant overlapping of the different age categories of birds the virus has spread. This is supported by the fact that in the same year, outbreaks were declared mainly in administrative areas with high population density of mule ducks, namely Plovdiv, Haskovo, Stara Zagora and Dobrich. The first three are geographically connected.

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