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Original Contribution

DIAGNOSTIC PROCEDURE FOR DETECTION OF HPAI H5N8-BULGARIAN EXPERIENCE DURING THE EPIZOOTIC WAVE IN 2016/2017

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ABSTRACT

In the 4 months spanning the 2016/2017 winter (October – January), HPAI H5N8 was the predominant serotype throughout European countries. Bulgaria, an important geographical location for migratory birds crossing Europe through the two major migratory flyways for Africa, was heavily affected with HPAI H5N8 outbreaks. The first detection of HPAI virus serotype H5N8 in Bulgaria was on the 19th of December 2016 in the Vidin region. Subsequently many outbreaks in wild birds and domestic poultry were reported in 15 different administrative regions. By the end of January 2017, the HPAI H5N8 strain was detected in domestic poultry, game birds, wild waterfowl, and zoo birds. The observed and reported symptoms were discoordination, laboured breathing, ataxia, opisthotonos, watery diarrhoea, sudden death, high mortality, weakness, and recumbency. In wild birds data for high mortality was only available for some species, with well-defined hyperaemia of the meninges and brain congestion with a singular haemorrhage being mainly observed, particularly in Dalmatian pelicans. These observations showcase the importance of rapid and accurate detection and subtyping of these HPAI viruses by research laboratories. To tackle this issue, the National Reference laboratory for Influenza A and Newcastle disease in Sofia adapted a modified real-time RT-PCR assay for detection of the N8 subtype in domestic and wild birds. The aim of this study is to present a Bulgarian experience in diagnosis of HPAI H5N8 subtype during 2016/2017 epizootic wave.

Key words: Influenza, wild birds, domestic poultry, Bulgaria, diagnosis

INTRODUCTION

Highly pathogenic avian influenza (HPAI) subtype H5N8 was first detected in Europe in a Eurasian wigeon (*Anas penelope*) in Russia in September 2014 (1). Subsequently, from November 2014 until February 2015, HPAIV H5N8 clade 2.3.4.4 spread widely across Europe, and by the end of October 2016, gave rise to the largest bird flu epidemic in Europe. By the middle of January 2017 the viral spread resulted in 974 outbreaks being registered in many European countries.

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Viral reassortment, the process of genetic recombination is a common phenomenon particularly in influenza viruses. Reassortment of the H5 subtype with different low pathogenic avian influenza (LPAI) strains have been found to circulate in wild waterfowl. In the 2016/2017 epidemic period, the most prevalent subtype within the European territory was H5N8, although HPAI of different subtypes were also detected (2). More recently, between September and December 2021, 27 European countries reported 867 outbreaks of HPAI H5N8 (316 in domestic poultry, 523 in wild birds, and 28 in captive birds). The most affected countries with outbreaks in domestic poultry were Italy with 167, followed by Poland and Hungary with 35

each. In wild birds the most detections were reported by Germany, the Netherlands, and the United Kingdom (3). The geographical location of Bulgaria makes it an important migration spot for migratory birds crossing Europe via the two main migratory pathways for Africa. The 2016/2017 epidemic season in Bulgaria began with an outbreak in a backyard farm in the Vidin region on the 19th of December, 2016. Subsequently an additional 15 different administrative regions of the country became affected, the results of which were the largest ever avian influenza epizooty in the history of the country (4). Bulgaria is one of the biggest producers of foie gras in Europe, with many farms with fattening ducks in and around the Plovdiv and Haskovo regions. Many of these were affected by HPAI H5N8 during the 2016/2017 epidemic season, with thousands of birds being diagnosed and subsequently destroyed through a mandated national program for eradication and control of HPAI. The next epizootic wave in 2017/18 did not spare Bulgaria and the country continued to report outbreaks (5). One recent study analyzed 16 newly sequenced H5 viruses detected in the country between 2019 and 2021, describing their genetic diversity and the emergence of a new H5N2 subtype, likely generated from a local reassortment event (6). Rapid and accurate detection and subtyping of these HPAI viruses is one of the most important priorities for laboratories. The real time reverse transcriptase polymerase chain reaction (rRT-PCR) assay is a sensitive and powerful technique for the detection of viruses and their subtypes. Hoffmann and colleagues (7) described a rapid and modifiable rRT-PCR method for specific detection of neuraminidase (NA) subtypes of avian influenza in global circulation. The National Reference laboratory for Influenza A and Newcastle disease in Sofia modified the described method for detection of N8 in the HPAI circulating in Bulgaria with the main goal for a rapid and accurate detection and subtyping of these HPAI viruses

MATERIALS AND METHODS

Samples. Sampling period was a part of national surveillance program for Avian influenza in domestic poultry and wild birds during the epizootic wave from November 2016 to the end of January 2017. Analysis for avian influenza A was performed on cloacal and tracheal swabs, organ suspensions (intestine and lungs) and fresh feces. A 10% suspension (w/v) of ground sample was prepared in Minimum Essential Media (MEM, pH 7,2-7,4) supplemented with Streptomycin (200 mg/L), Penicillin G ($2x10^6$ IU/L), Nystatin dehydrate ($0,5x10^6$ IU/L), Polymyxin B ($2x10^6$ IU/L), Gentamicin sulfate (250 mg/L), and Sulphamethoxazole (200 mg/L). Microbiological testing was performed on each sample to exclude bacterial infection as cause of mortality.

RNA extraction. Nucleic acid extraction from tissue homogenate supernatant was performed using the *QIAamp Viral RNA Mini Kit (QIAGEN, Germany)* and the *High pure RNA isolation kit (Roche Diagnostics, Germany)* according to manufacturer's instructions.

Polymerase chain reaction (PCR). For real time reverse transcription polymerase chain reaction, the *AcuFlock® Influenza A Virus real time RT-PCR Kit* - for M gene and the QIAGEN One-Step Kit *(QIAGEN, Germany)* were used. The *Virotype mix+IC(TAMRA)-RNA* amplification kit was used for H5 detection as previously described (8). AgPath-ID One-Step RT-PCR reagents (*Thermo Fischer scientific, USA*) were used for detection of N8 AIVs in global circulation per RITA analysis as described by Hoffmann et al., 2016 (7). Volumes of primers and probes were reduced to 2 µl at the expense of water, and the reaction time in the second amplification step was increased to 40 seconds. **H5-kha**-1 and **H5-kha**-3 primers for conventional H5 PCR were used (9). The sequences of the specific primers and probe and the temperature regime of the reactions for real time and conventional PCRs are shown in **Table 1.**

Virus isolation and identification. Samples from each bird were centrifuged at 800g for 10 minutes at 4°C after homogenization. Inoculation into the allantoic cavity of three 9-day-old chick embryos was then performed using 200µl of each sample supernatant as specified in the OIE Terrestrial Manual (Chapter 3.3.4, 2021). Subsequently, allantoic fluids were tested for hemagglutination activity via the hemagglutination assay (HA assay). The HA

positive fluids were tested via hemagglutination inhibition (HI) using 4 hemagglutination units and hyperimmune serum (APMV1 (NDV), H5N1, H5N8, H7N1) produced by the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe, Italy). For both assays (HA and HI), standard OIE procedure was followed (OIE Terrestrial Manual, Chapter 2.3.4, 2015).

Table 1. *Sequences of the specific primers and probe and the temperature regime of the performed rRT-PCR and conventional PCR.*

	N8	H5 rRT-PCR	H5 PCR
Primers	**IVA-N8-1296F** TCC ATG YTT TTG GGT TGA RAT GAT	ACA TAT GAC TAC CCA CAR TATTCGA	**H5-kha**-1: CCT CCA GAR TAT GCM TAY AAAATT GTC
Primers	**IVA-N8-1423R** GCT CCA TCR TGC CAY GAC CA	AGA CCA GCT AYC ATG ATT GC	**H5-kha**-3: TAC CAA CCG TCT ACC ATK CCY TG
Probes	**IVA-N8-1354FAM**-TCH AGY AGC TCC ATT GTR ATG TGT GGAGT-**BHQ1**	**FAM**-TCWACA GTG GCG AGT TCC CTAGCA -**TAMRA**	
Temperature regime	50°C – 30 min	50°C – 30 min	50°C – 30 min
	95°C – 15 min	95°C – 15 min	95°C – 15 min
	45 cycles	**40 cycles**	**35 cycles**
	95°C – 15 s	95°C – 15 s	94°C – 30 s
	56°C – 40 s	54°C – 60 s	58°C – 30 s
	72°C – 30 s		68°C – 60 s
			Extension 68°C – 7 min

RESULTS

Until the end of January 2017, the HPAI H5N8 strain was detected in domestic poultry including ducks, geese, and guinea fowl, as well as in zoo and game birds. The virus was also detected in wild birds, which included the Dalmatian pelican *(Pelecanus crispus)*, Long-eared owl *(Asio otus)*, Swan *(Cygnus olor)*, Eurasian wren *(Troglodytes troglodytes)*, Swan goose *(Anser cygnoides)*, Eurasian coot *(Fulica atra)* and Great Egret *(Ardea Alba)* bird species. The observed and reported symptoms were discoordination, labored breathing, ataxia, opisthotonus, watery diarrhea, sudden death, high mortality, weakness, and recumbency. In the wild birds data for high mortality was only available for some species.

Necropsy findings. Most prominent changes were observed in gallinaceous birds (guinea fowls, chickens, and pheasants). Petechial hemorrhages were found on the cuticula of the muscular stomach and on the serosal surfaces of the lungs and spleens of the affected birds. Diffuse hemorrhages around the cecal tonsils were also observed **(Figure 1a)**. Small point necrosis, hemorrhages and red-brown spots were observed on the pancreas (**Figure 1b**, arrows). In the affected wild birds, a few macroscopic changes were observed only in the Dalmatian pelican. Well defined hyperemia of the meninges and brain congestion with singular petechial hemorrhages **(Figure 1c).** Gross pathologic changes were not observed in the lungs, heart, liver, spleen, and kidney.

Polymerase chain reaction. All submitted samples were tested with the AcuFlock® Influenza A Virus real time RT-PCR Kit. Samples positive for M gene segments were further tested for H5 and N8 **(Figure 2)** using adapted conditions as outlined above, as well as with conventional H5 PCR **(Figure 3).** Samples with positive result in the conventional H5 PCR were purified and sent for sequencing. Identification of the multiple basic amino acids (Arginine (R) and Lysine (K)) at the cleavage site of HA was then established to confirm the pathogenicity of the detected viruses.

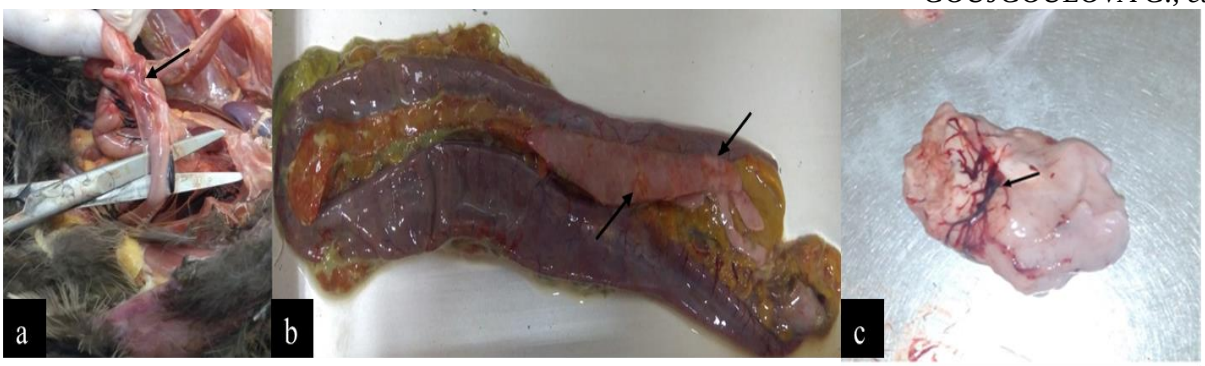

Figure 1. a- caecum, showing diffuse sub-serosal haemorrhages; **b- pancreas,** showing necrosis and petechial hemorrhages, and streaked with red/brown spots of varying sizes (arrows); **c- brain,** showing hyperemia and congestion.

Setup | Instrument | **Results**

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | **Report**

Well	Sample Name	Detector	Task	Ct
A1	pos.-3	AI N8	Unknown	19.08
A2	pos.-2	AI N8	Unknown	18.33
B1	ntc	AI N8	NTC	Undet.
B2	ntc	AI N8	NTC	Undet.
C1	ter.proba	AI N8	Unknown	33.79
C2	ter.proba	AI N8	Unknown	31.71

Figure 2. Positive samples for N8, rRT-PCR.

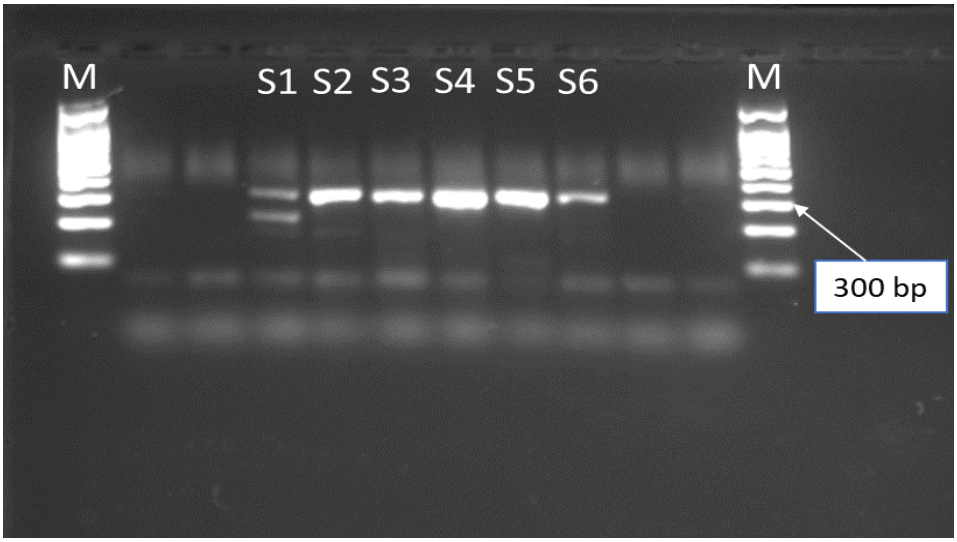

Figure 3. Gel electrophoresis visualizes the results of samples with the size of the product when working with kha H5 primers. M indicated molecular weight markers. S designates samples 1 through 6. Arrow indicates 300 base pair.

Virus isolation and subtyping. All PCR positive samples were inoculated into 9-days-old embryonated chicken eggs. The virus killed the embryos after 24-48 h following organ suspension inoculation into the allantoic cavity of embryonated chicken eggs, in all cases of H5N8 isolation. After inspection, multiple petechial hemorrhages throughout the embryo's body were

observed. The allantoic fluid of the dead embryos was tested for hemaglutination activity with the HA assay, with positive samples subsequently assayed with HI, following the standard operating procedure previously outlined. We found that all isolated viruses were subtype H5, with all positive samples showing a high titer for H5N8 and a low or negative titer for H5N1.

DISCUSSION

Bulgaria is a favorite wintering ground for a large number of wild migratory birds crossing Europe. In early winter each year, thousands of migrating waterfowl use Bulgaria as a resting stop or wintering area before they continue their journey southward. Preliminary data from the 41st Midwinter Census of Waterfowl in Bulgaria, held from 13 to 15 January 2017, showed some of the highest numbers established in recent years. Among the large flocks of wintering waterfowl in the Burgas Lakes region, four pink flamingos were identified, which are rare visitors in Bulgaria. Wintering geese were also observed in large numbers by the Bulgarian sea coast, including the great white-fronted goose, the globally endangered, red-breasted goose, and small white-fronted geese, which are a very rare species seen in the country. A large number of migratory birds, exotic species, and the severe epizootic situation throughout Europe with regard to Influenza A viruses have contributed to the widespread of HPAI H5N8 in Bulgaria.

The reported clinical signs in Europe in chickens during the 2016/2017 period affected by HPAIV A(H5N8) were variable, and consisted of nervous signs including head shaking, ataxia, tremors, diarrhea and poor general condition. On some holdings, chickens died suddenly without prior clinical signs (10). We also found examples of sudden death in our studies in gallinaceous bird species because of HPAI H5N8 infection. In the affected wild birds only hyperemia of the meninges and brain congestion with singular petechial hemorrhages were found in Dalmatian pelicans. The observed brain congestion and hyperemia in our case in this bird species suggest viral neurotropism. The neurotropism, and consequently, neurological disease of these HPAI viruses in wild bird species, is described in previous studies (11, 12). Earlier studies showed that HPAI viruses in some wild bird species are highly neurotropic and cause severe neurological disease (13). The mechanisms of dissemination of the virus in the brain in wild birds are not yet fully understood. However, the explanation that the route of infection also affects the route of dissemination is plausible. The clinical and pathological findings reported by authors from other European countries overlap with those found in Bulgaria. In this study serious attention has also been paid to the adaptation of molecular biological diagnostic methods, at the same time, understanding their importance and indispensability, the gold standard methods for etiological and virological diagnosis of Avian Influenza were also used. Successful optimization, adaptation and implementation of protocols for rRT-PCR for N8 and conventional PCR are ones of the most important priorities for the National Reference laboratory for Avian influenza and Newcastle disease. The rapid detection, subtyping and pathotyping of those highly infectious viruses are possible with these molecular methods. It is a rapid (compared with the gold standard methods: virus isolation, HA and HI) method, allowing a proof of Influenza A virus and determining their subtypes and pathotypes. Such allows ease of deployment and year-round use for the execution of the State preventive surveillance program of Influenza A in wild and domestic birds in Bulgaria. The characterization of the prevalent strains in the wild and domestic bird populations in Bulgaria with the help of molecular biological technics (rRT-PCR, conventional PCR, sequencing and Phylogenetic analysis) would answer the questions about the origin and evolution of these viruses, and would be extremely useful for epidemiological surveillance and analysis.

CONCLUSION

Rapid and accurate detection and subtyping of HPAI viruses are one of the most important priorities for laboratories. The National Reference laboratory for Influenza A and Newcastle disease in Sofia was able to identify and analyze viral infection in domestic and wild birds by adapting the previously reported Riems influenza A typing array (RITA) rRT-PCR method to detect N8.

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