ISSN: 1220-3173; E-ISSN: 2457-7618

ROMANIAN ACTIVE SURVEILLANCE OF AVIAN INFLUENZA VIRUS INFECTIONS IN WILD BIRDS: RETROSPECTIVE ANALISIS 2018-2019

SUPRAVEGHEREA ACTIVĂ A INFECȚIILOR CU VIRUSURILE GRIPEI AVIARE LA PĂSĂRILE SĂLBATICE DIN ROMÂNIA: ANALIZĂ RETROSPECTIVĂ 2018-2019

Florica BĂRBUCEANU[1),2),*)], Raluca BURLACU[1)], Iuliana ONITA[1)], Gabriela Florina MATEI[1)], Paula TAMBA[1)], Nicoleta FOLTOS[1)], S. BĂRĂITĂREANU[2)]

ABSTRACT | REZUMAT

Over 100 bird species proved to be susceptible at AIV infection. In the epidemiology of AIV, wild birds are acting as the reservoir of AIVs and are the main source of virus transmission to domestic birds. To reduce the risk of transmitting AIVs from wild birds to farm birds, passive and active surveillance programs are implemented to detect early the introduction of the virus in a certain area. The aim of the study is the retrospective analyses of AIV H5/H7 active surveillance in Romanian wild birds in 2018 and 2019. During the analysed period, all molecular tests (482 samples) performed for AIV Matrix protein detection in wild birds (44 species) from Romanian provided negative results.

Keywords: Avian influenza virus, PCR, wildlife, surveillance

Peste 100 de specii de păsări s-au dovedit a fi susceptibile la infecția cu AIV. În epidemiologia AIV, păsările sălbatice acționează ca rezervor de AIV și sunt principala sursă de transmitere a virusului la păsările domestice. Pentru reducerea riscului transmiterii AIVs de la păsările sălbatice la păsările de fermă, sunt implementate programe de supraveghere pasivă și activă care să detecteze timpuriu introducerea virusului într-un anumit areal. Scopul acestei lucrări este analiza retrospectivă a supravegherii active a AIV la păsările sălbatice din România în anii 2018 și 2019. În perioada analizată, toate testele moleculare (482 probe) efectuate pentru detecția proteinei Matrix a virusului gripei aviare la păsările sălbatice (44 de specii) din România au furnizat rezultate negative.

Cuvinte cheie: Avian influenza virus, PCR, animale sălbatice, supraveghere

Avian Influenza viruses (AIVs) are a group of various strains of influenza A virus recognised to infect many species of birds, both wild and domestic, and occasionally humans (10). AIVs are part of the *Orthomyxoviridae* family in genus *Influenzavirus A* and are splits into subtypes based on the haemagglutinin and neuraminidase antigens (1, 2). The name of the AIV subtype describes the types of the haemagglutinin and neuraminidase antigens combination (e.g., H7N9, H5N1, and H10N8) (10). To date, the most well-known strains involved in human infection are H5N1 and H7N9 (6, 10). AIVs are classified in two pathogenic groups: highly pathogenic avian influenza (HPAI) viruses and low pathogenic avian influenza (LPAI) viruses (1). HPAI strains cause an extremely severe systemic disease with very high flock mortality, while LPAI strains cause a mild respiratory disease in the absence of other co-infections or favouriting factors (2).

AIVs have been isolated from over 100 species, but not all play the same role in the virus epidemiology (5). Several *Anseriformes* (e.g. ducks, geese, swans) and *Charadriiformes* (e.g., shorebirds) species are thought to act as the reservoir for AIV (13). The wild birds are the primary reservoir of AIVs transmission to domestic poultry (11), while domestic poultry are usually responsible for human disease outbreaks (10).

The surveillance programme for avian influenza in wild birds applied by European Union member states has as the main objective "the timely detection of HPAI of the subtype H5N1 in wild birds in order to protect poultry in poultry holdings and safeguard veterinary public health". Also, considering the Commission decision of 25 June 2010 on the implementation by Member States of surveillance programmes for avian influenza in poultry and wild birds, notified under document C (2010)4190(2010/367/EU), the wild birds testing shall be carried out at the NRL in Member States or other laboratories authorised by the Romanian competent authorities and under the control of the NRL located at the Institute for Diagnosis and Animal Health (3).

The aim of the study is the retrospective analyses of AIV active surveillance in Romanian wild birds in 2018 and 2019.

1) Institute for Diagnosis and Animal Health, Bucharest, Romania
2) University of Agronomic Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Bucharest, Romania
*) *Corresponding author email: florica.barbuceanu@idah.ro*

MATERIALS AND METHODS

All procedures, sampling methods and criteria for the evaluation of the results of laboratory tests were set out in accordance with the European Union guidelines and regulations and carried out in a biosafety level 2 facility at the IDSA (2).

Samples

A total of 482 wild-birds were included in the national active surveillance program in Romania in 2018 and 2019 (Table 1). Sampling was performed by Institute for Diagnosis and Animal Health or Sanitary Veterinary and Food Safety Directorates. From captured birds oropharyngeal and cloacal swabs were collected, while whole carcasses, oropharyngeal and cloacal swabs were harvested from the corpses.

Table 1

Wild bird tested in Romania for AIV H5/H7 in 2018 and 2019

No.	County	No. wild birds		
		2018	2019	Total
1.	Alba	0	0	0
2.	Arad	7	4	11
3.	Arges	2	8	10
4.	Bacau	10	6	16
5.	Bihor	0	10	10
6.	Bistrita-Nasaud	10	10	20
7.	Botosani	0	0	0
8.	Brasov	8	15	23
9.	Braila	10	20	30
10.	Buzau	0	0	0
11.	Caras-Severin	10	10	20
12.	Calarasi	7	22	29
13.	Cluj	12	18	30
14.	Constanta	4	2	6
15.	Covasna	10	7	17
16.	Dambovita	13	12	25
17.	Dolj	52	9	61
18.	Galati	2	2	4
19.	Giurgiu	0	0	0
20.	Gorj	0	0	0
21.	Harghita	0	0	0
22.	Hunedoara	0	0	0
23.	Ialomita	7	6	13
24.	Iasi	0	2	2
25.	Ilfov	1	0	1
26.	Maramures	0	1	1
27.	Mehedinti	0	0	0
28.	Mures	0	8	8
29.	Neamt	1	1	2
30.	Olt	0	7	7
31.	Prahova	15	10	25
32.	Satu-Mare	11	10	21
33.	Salaj	7	10	17
34.	Sibiu	4	0	4
35.	Suceava	0	1	1
36.	Teleorman	0	0	0
37.	Timis	0	0	0
38.	Tulcea	32	10	42
39.	Vaslui	0	10	10
40.	Valcea	0	0	0
41.	Vrancea	9	0	9
42.	Bucuresti	6	1	7
Total		**250**	**232**	**482**

RNA extraction

Total RNA was isolated according the manufacturer's instructions, with the following commercial kits HighPure RNA Isolation Kit (Roche,Germany), RNeasy Mini kit (Qiagen, USA), Nucleospin RNA II (Macherey–Nagel, Germany), and Pure Link RNA mini kit (Invitrogen, USA).

Real time reverse-transcription PCR (**real-time RT-PCR**)

Real time RT-PCR was performed with the commercial kits OneStep RT-PCR (Qiagen, USA), Superscript III One Step RT-PCR with Platinium Taq polymerase (Invitrogen, USA), and Ag Path One Step RT-PCR (Invitrogen, USA) following the manufacturer's instructions.

Primers and probe used in matrix protein detection were Matrix Forward: CTT CTA ACC GAG GTC GAA ACG TA, Matrix Reverse: CAC TGG GCA CGG TGA GC and Taq1: 5'–FAM-CTC AAA GCC GAG ATC GCG CAG A.

All Real Time RT-PCR tests for detection of AIVs were found with negative results.

The strategy of the active surveillance programmes in detection of AIVs when we deal with positive Matrix gene result is to identify the H5 or /and H7 subtypes by Real Time RT-PCRs recommended by EURL, and to establish the category of HPAI or LPAI strains by conventional RT-PCRs and sequencing reaction of the cleavage site of hemagglutinin gene.

Matrix protein detection with OneStep RT-PCR (Qiagen, USA) was performed using Light Cycler (Roche, Germany) and Light Cycler Software 4 with the following cycling parameters: 50°C – 30', 1x; 95°C – 15', 1x; 95°C – 10'', 58°C – 40'', 72°C – 30'', 45x; 40°C – 30'', 1x.

Matrix protein detection with Superscript III One Step RT-PCR with Platinium Taq polymerase (Invitrogen, USA) used the following cycling parameters: 55°C – 30', 1x; 95°C – 5', 1x; 95°C – 10'', 54°C / 54°C – 30'', 68°C – 30'', 45x; 40°C – 30'', 1x.

The limit of detection of the Matrix protein detection test by Real Time RT-PCR have to be establish very carefully depending on reagents, biological material and also instruments. In the Molecular Biology Department from IDAH, samples with Ct ≤35were considered positive, samples within Ct 35 and Ct 38 should be repeated starting form original material, and samples over Ct 38 were considered negative.

RESULTS AND DISCUSSIONS

The tests performed in 2018 covered 32 different species of wild birds (Table 2) from 24 counties, as follows: Arad, Arges, Bacau, Bistrita-Nasaud, Brasov, Braila, Caras-Severin, Calarasi, Cluj, Constanta, Covasna, Dambovita, Dolj, Galati, Ialomita, Ilfov, Neamt,

Table 2

Species of wild bird tested in Romania for AIV matrix protein detection in 2018 and 2019

No.	*Scientific name*	2018	2019	Total
1.	*Accipiter gentilis* (Northern Goshawk)	0	1	1
2.	*Accipiter nisus* (Eurasian Sparrowhawk)	2	1	3
3.	*Anas clypeata* (Northern Shoveler)	0	3	3
4.	*Anas crecca* (Common Teal)	11	10	21
5.	*Anas platyrhynchos* (Mallard)	35	54	89
6.	*Anas stepera* (Gadwall)	0	2	2
7.	*Anser albifrons albifrons* (Greater White-fronted Goose)	0	2	2
8.	*Anser brachyrhynchus* (Pink-Footed Goose)	2	0	2
9.	*Anser erythropus* (Lesser White-Fronted Goose)	0	1	1
10.	*Ardea cinerea* (Grey Heron)	0	3	3
11.	*Bubo bubo* (Eurasian Eagle-Owl)	2	0	2
12.	*Buteo buteo* (Common Buzzard)	2	2	4
13.	*Ciconia ciconia* (White Stork)	7	9	16
14.	*Circus aeruginosus* (Eurasian Marsh Harrier)	2	0	2
15.	*Columba liva* (Rock Dove)	2	0	2
16.	*Corvus corone* (Carrion crows)	2	2	4
17.	*Corvus frugilegus* (Rook)	3	0	3
18.	*Corvus monedula* (European Jackdaw)	3	0	3
19.	*Cygnus atratus* (Black Swans)	2	0	2
20.	*Cygnus cygnus* (Whooper Swan)	5	5	10
21.	*Cygnus olor* (Mute Swan)	9	1	10
22.	*Dendrocopos syriacus* (Syrian Woodpecker)	1	0	1
23.	*Falco tinnunculus* (Common Kestrel)	0	6	6
24.	*Fulica atra* (Eurasian Coot)	18	4	22
25.	*Gallinago gallinago* (Common Snipe)	1	0	1
26.	*Garrulus glandarius* (Eurasian Jay)	4	1	5
27.	*Haliaeetus albicilla* (White-Tailed Eagle)	0	1	1
28.	*Ixobrychus minutus* (Little Bittern)	0	1	1
29.	*Larus canus* (Common Gull)	6	1	7
30.	*Larus ridibundus* (Black-Headed Gull)	2	0	2
31.	*Larus sp.* (Gull)	1	2	3
32.	*Limosa limosa* (Black-Tailed Godwit)	1	4	5
33.	*Mergus albellus* (Smew)	0	5	5
34.	*Milvus migrans* (Black Kite)	6	11	17
35.	*Milvus milvus* (Red Kite)	1	2	3
36.	*Passer Montanus* (Eurasian Tree Sparrow)	1	0	1
37.	*Phalacrocorax carbo* (Great Cormorant)	3	6	9
38.	*Phasianus colchicus* (Common Pheasant)	0	4	4
39.	*Pica pica* (Eurasian Magpie)	102	84	186
40.	*Porphyrio porphyrio* (Purple Swamphen)	4	0	4
41.	*Sterna hirundo* (Common Tern)	0	4	4
42.	*Streptopelia decaocto* (Eurasian Collared Dove)	3	0	3
43.	*Sturnus vulgaris* (European Starling)	4	0	4
44.	*Tachybaptus ruficollis* (Little Grebe)	3	0	3
Total		**250**	**232**	**482**

Prahova, Satu-Mare, Salaj, Sibiu, Tulcea, Vrancea, and Bucharest. In 2019, there were analysed samples from 29 different species of wild birds and 28 counties, as follows: Arad, Arges, Bacau, Bihor, Bistrita-Nasaud, Brasov, Braila, Caras-Severin, Calarasi, Cluj, Constanta, Covasna, Dambovita, Dolj, Galati, Ialomita, Iasi, Maramures, Mures, Neamt, Olt, Prahova, Satu-Mare, Salaj, Suceava, Tulcea, Vaslui, and Bucharest.

Despite of high number of wild bird species included in active surveillance of AIVs in wildlife in 2018 and 2019, the surveillance is far from covering the diversity of wild birds or their abundance at the county and national level. The Romanian avifauna included 402 confirmed species (7), and in our study were covered only 10.94% (44/402) of them. Moreover, only one species (*Pica pica*) was sampled >80 times in each year.

In Fig. 1 is described a graphic of 15 negative samples of wild bird's species of *Pica pica* in Dolj County by Real time RT-PCR Matrix protein detection test.

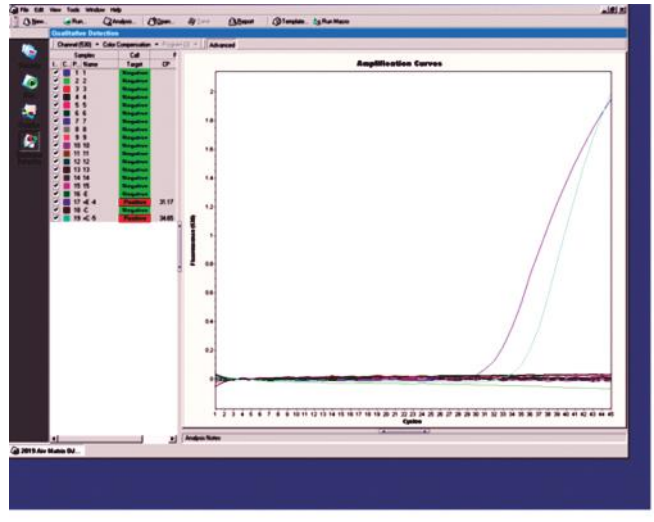

Fig. 1. Graphic representation of a Real Time RT-PCR –Matrix protein detection test with clearly negative amplification curves in all 15 samples: 1-15-negative samples; -E-negative extraction control; +E- positive extraction control; -C- negative amplification control; +C – positive amplification control

In our lab, the positive controls are periodically tested to be sure that it is fit for purpose of the method. In Fig. 2 it is represented a dilution series of an inactivated viral strain of H5N8.

Considering the current active surveillance of avian influenza virus in Romanian wild birds, the low number of species and samples tested in each year may be related to the opportunistic way of obtaining samples. A similar situation has been reported in previous surveys (8, 12). In the active surveillance of AIVs in wildlife is important to understand the host ecology before understood the ecology, epidemiology, genetics, and evo-

lution of the virus (8), and that recommends rethinking of the current surveillance infrastructure and strategy, at least in the sense of increasing the diversity of the tested species and of correlating the number of samples with the density of wild birds in the area at the time and location of sampling. Hoye et al. (2010) states that there is a high risk of bias toward species that is easily caught or is present in accessible areas at high concentrations (5). Moreover, Peterson et al. (2008) and Gronesova et al. (2007) reported that a high proportion of samples from eight different passerine families show infection when sampled in or near waterfowl-rich bodies of water (4, 9). Consequently, active surveillance may not detect early outbreaks in the wildlife and the virus will be able to be transmitted to domestic birds and later to humans.

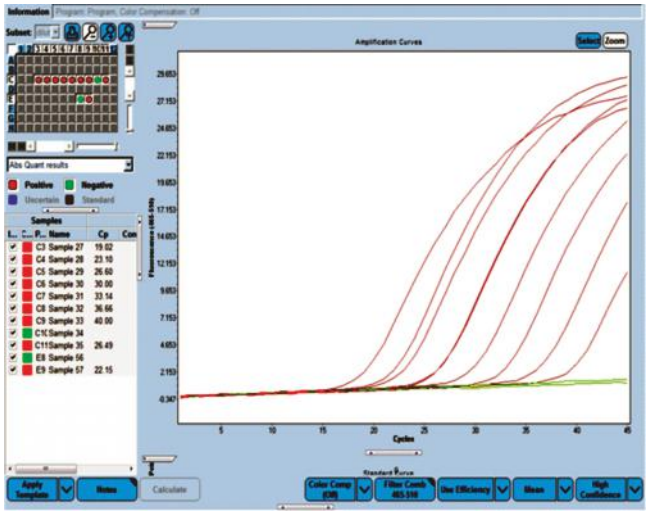

Fig. 2. Amplification curves obtained by serial dilution of H5N8 inactivated strain beginning from 10^{-1} to 10^{-7} ten-fold dilutions; C10- negative extraction control; C11- positive extraction control; E8 – negative amplification control; E9 – positive amplification control

CONCLUSIONS

In 2018 and 2019, all molecular tests (482 samples) performed for AIV H5/H7 in Romanian wild birds (44 species) provided negative results. The result may not reflect the real situation of AIVs circulation in wildlife and rethinking of the current surveillance program for AIV in wild birds should be considered. This decision should take into account the real risk of transmitting AIVs from wild to domestic birds, especially during periods when the population and diversity of migratory bird species is increasing.

REFERENCES

1. *Alexander D.J.,*(2007), An overview of the epidemiology of avian influenza. Vaccine, 25(30):5637-5644
2. *European Commission,* (2006), 2006/437/EC: Commission Decision of 4 August 2006 approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC. OJ L (118M, 8.5.2007):1285-1311
3. *European Commission,* (2010), 2010/367/: Commission Decision of 25 June 2010 on the implementation by Member States of surveillance programmes for avian influenza in poultry and wild birds (notified under document C(2010) 4190) (Text with EEA relevance). OJ L, 166: 22-32
4. *Gronesova P., Kabat P., Trnka A., Betakova T.,* (2008), Using nested RT-PCR analyses to determine the prevalence of avian influenza viruses in passerines in western Slovakia, during summer 2007. Scandinavian journal of infectious diseases, 40(11-12):954-957
5. *Hoye B.J., Munster V.J., Nishiura, H., Klaassen M., Fouchier R.A.,* (2010), Surveillance of wild birds for avian influenza virus. Emerging infectious diseases, 16(12):1827-1834
6. *Lazarus R., Lim P.L.,* (2015), Avian influenza: recent epidemiology, travel-related risk, and management. Curr Infect Dis Rep, 17(1):456
7. *Marton A.K.,* (2020), Romania Bird Checklist. iGo Terra. Available at: https://igoterra.com/country/checklist/119/taxagroup?group=33&filter=1&hideVagrants=false [Accessed: August 30, 2020]
8. *Olsen B., Munster V.J., Wallensten A., Waldenström J., Osterhaus A.D., Fouchier R.A*., (2006), Global patterns of influenza a virus in wild birds. Science (New York, N.Y.), 312(5772):384-388
9. *Peterson A.T., Bush S.E., Spackman E., Swayne D.E., Ip H.S.,* (2008), Influenza A virus infections in land birds, People's Republic of China. Emerging infectious diseases, 14(10):1644-1646
10. *Sendor A.B., Weerasuriya D., Sapra A.,* (2020), Avian Influenza. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available at: https://www.ncbi.nlm.nih.gov/books/NBK553072/ [Accessed: June 3, 2020]
11. *Spackman E.,* (2009), The ecology of avian influenza virus in wild birds: what does this mean for poultry ? Poultry science, 88(4):847-850
12. *Stallknecht D.E., Brown J.D*., (2008), Ecology of avian influenza in wild birds. In: Swayne DE, editor. Avian influenza, (Ed.) Blackwell Publishing, Ames, IA, USA, 43-58
13. *Webster R.G., Bean W.J., Gorman O.T., Chambers T.M., Kawaoka Y.,* (1992), Evolution and ecology of influenza A viruses.Microbiological reviews, 56(1): 152-179.