

***Original Article***

## **Surveillance of Highly Pathogenic Avian Influenza Viruses (H5Nx Subtypes) in Wild Birds in Iran, 2014-2019**

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Received 10 February 2020; Accepted 16 September 2020

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### **Abstract**

After the emergence of the highly pathogenic avian influenza viruses (HPAIV) subtypes H5N6 in 2013 and H5N8 in 2014, a surveillance study using molecular epidemiology approaches was carried out during 2014 – 2019 in Iran to discover any potential introduction or outbreak of HPAIV in wild bird populations. All sick and dead wild birds found in nature, or in cases of an outbreak, a collection of representative samples was tested using the specific molecular methods for HPAIV H5 subtypes. Additionally, wild bird species in wetlands, several zoos, zoological gardens, or rehabilitation centers were tested for HPAIV. During the active surveillance plan, several individual and outbreak cases of HPAIV and orthoavulaviruses were identified. In general, more than 900 fecal materials, cloacal and oropharyngeal swabs, and/or tissue samples were collected from apparently healthy live birds representing several different species and families. In addition, tissue and swab samples were collected and investigated from any reported wild birds' mortality cases in different parts of Iran in the framework of this study. No positive bird was found among apparently healthy live birds; however, the highly pathogenic influenza viruses of H5N1, H5N2, H5N6, and H5N8 were found in individual dead birds or mass die-off cases.

**Keywords:** highly pathogenic avian influenza viruses (HPAIV), surveillance, wild birds, outbreak, Iran

### **Surveillance des Virus de la Grippe Aviaire Hautement Pathogènes (Sous-Types H5Nx) chez les Oiseaux Sauvages en Iran, 2014-2019**

**Résumé:** Après l'émergence des sous-types de virus de la grippe aviaire hautement pathogène (VIAHP) H5N6 en 2013 et H5N8 en 2014, une étude de surveillance utilisant des approches d'épidémiologie moléculaire a été menée en 2014-2019 en Iran pour découvrir toute introduction ou épidémie potentielle de virus VIAHP dans les populations d'oiseaux sauvages. Tous les oiseaux sauvages malades et morts trouvés dans la nature, ou en cas d'épidémie, une collection d'échantillons représentatifs a été testée en utilisant les méthodes moléculaires spécifiques pour les sous-types VIAHP H5. De plus, des espèces d'oiseaux sauvages dans les zones humides, plusieurs zoos, jardins zoologiques ou centres de réhabilitation ont été testées pour le VIAHP. Au cours du plan de surveillance active, plusieurs cas individuels et épidémiques de VIAHP et d'orthoavulavirus ont été identifiés. En général, plus de 900 matières fécales, écouvillonnages cloacaux et oropharyngés et/ou échantillons de tissus ont été prélevés sur des oiseaux vivants apparemment en bonne santé représentant plusieurs espèces et familles

différentes. En outre, des échantillons de tissus et d'écouvillons ont été collectés et étudiés à partir de tous les cas de mortalité d'oiseaux sauvages signalés dans différentes parties de l'Iran dans le cadre de cette étude. Aucun oiseau positif n'a été trouvé parmi les oiseaux vivants apparemment sains; cependant, les virus grippaux hautement pathogènes H5N1, H5N2, H5N6 et H5N8 ont été trouvés dans des oiseaux morts individuels ou dans des cas de mortalité massive.

**Mots-clés:** virus de la grippe aviaire hautement pathogène (VIAHP), surveillance, oiseaux sauvages, épidémie, Iran

## 1. Introduction

Wild waterbirds are considered the main reservoir of low pathogenic avian influenza viruses (LPAIV), and those viruses are widely distributed in wild avian species around the world. They have been most frequently identified in waterbirds of the orders Anseriformes (including ducks, geese and swans) and Charadriiformes (particularly gulls and terns). These viruses replicate in epithelial cells of the respiratory and intestinal tracts of birds and are excreted in high concentrations in their feces (1). It is now well recognized that global influenza virus surveillance in wild birds is important for understanding the role of wild birds in the epidemiology and ecology of these viruses.

Since the highly pathogenic avian influenza viruses (HPAIV) subtype H5N1 (A/Goose/Guangdong/1/1996) was first detected in China, many different novel viruses and subtypes of H5 have been generated due to genetic evolution and re-assortment with other avian influenza viruses (AIV). The HPAIVs, such as H5N2, H5N5, H5N6, and H5N8, have been detected worldwide and can infect wild birds, poultry, humans, and other species; they have been disseminated, at least partly, via wild birds (2, 3).

The HPAI H5N8 virus was first detected in live bird markets in China in 2010 (4). The H5N8 clade 2.3.4.4 virus (designated as clade 2.3.4.4a) was generated from that virus and contained the HA segment of H5 clade 2.3.4.4 and seven segments from other AIVs circulating in China (4). The first outbreaks of the HPAI H5N8 virus of clade 2.3.4.4 in domestic and wild birds were reported in January 2014 in South Korea (4), in April

2014 in Japan and Siberia (5), and by the end of 2014 and early 2015 in many European countries and North America. There is evidence for preliminary dissemination and transmission of the virus by migratory wild bird populations (6, 7).

In May–June 2016, a novel reassortant of H5N8 HPAI viruses (clade 2.3.4.4b) was detected in dead wild aquatic birds in Qinghai Lake. The virus spread to Mongolia, Siberia, and Europe and caused extensive mortality in wild birds and domestic poultry. Migratory water birds mixing with poultry may contribute to the generation of new viruses by reassortment and eventually give rise to intra- and intercontinental spread (7). Genetic analysis and animal experiments showed no indication of a zoonotic potential of H5N8a and b viruses related to the clade 2.3.4.4 (7).

After each expansion of HPAIV H5N1, H5N8, and H5N6 subtypes from Southeast Asia into many Eurasian and African countries, the frequency and intensity of avian influenza surveys in the world increased dramatically. North American and European countries, in particular, gathered massive epidemiological information regarding the circulation of AIVs in wild birds. Nonetheless, little is known about the prevalence of AIVs in wild birds in West & Central Asian and Middle Eastern countries. Many countries in this region were severely affected by each wave of HPAI outbreaks. Different subtypes of AIVs have been previously reported in wild birds and domestic poultry in Iran (8-11). The wetlands located in different parts of Iran, especially those in regions south of the Caspian Sea, represent major wintering and stopover sites during migration for many wild waterbirds from

Siberia and northern Russia. Several million migratory birds usually arrive in October and either remain until February/March or migrate further south (10). Here, we describe the results of four years of AIVs surveillance in wild birds in Iran, especially in areas south of the Caspian Sea in Iran.

## 2. Material and Methods

### 2.1. Sampling Plan and Diagnostic Procedures

A combination of active and passive surveillance studies was planned to investigate wild birds for highly pathogenic avian influenza infections in Iran. Samples were collected from 918 wild birds belonging to several different bird species (Tables 1, Table 2, and Table 3), mainly from dead birds, but also from zoological gardens, zoos, and bird rehabilitation centers, from 2014 to 2019 at 129 sites located in 27 provinces of Iran (Figure 1). The sampling periods were divided into three distinct times: pre-epidemics, during epidemics, and post-epidemics. The pre-epidemics wild birds surveillance study was conducted in January 2014 - January 2016 in five provinces, Gilan, Mazandaran, Golestan, West Azerbaijan, and Qom. In total, 268 cloacal and oro-pharyngeal samples from 134 wild birds were collected and investigated. Samples came from 34 avian species belonging to seven different families.

During the epidemic period, every reported mass or individual mortality of wild birds was investigated. The number and species of wild birds investigated in this period are presented in the results section.

After the last reported HPAIV-H5N6 outbreak in February 2018 in Bojaq National Park, the surveillance study continued in different provinces of the country, and approximately 160 dead wild birds were tested for HPAIV-H5.

Samples that were collected from dead birds included cloacal/oropharyngeal and tissue samples (liver, heart, spleen, and lungs). From healthy birds, only cloacal and oropharyngeal samples were collected. Cloacal and oropharyngeal samples were collected with cotton swabs, stored in viral transport medium (Hank's medium or PBS) containing antibiotics, and maintained at  $-70^{\circ}\text{C}$  after arrival at the laboratory. They were analyzed by a combination of conventional and real-time reverse transcription PCR (rRT-PCR). Positive samples were further confirmed by partial short fragment sequencing of the HA and NA genes.

### 2.2. RNA Extraction, RT-PCR, and Real-Time RT-PCR

Viral RNA was extracted using a QIAamp Viral RNA kit (Qiagen) for swab and tissue samples according to the manufacturer's instructions. The samples were tested by a modified TaqMan one-step real-time RT-PCR assay targeting the influenza A viruses matrix gene (12). Reverse transcription-PCR (RT-PCR) assays were performed based on one-step protocols using appropriate RT-PCR Kits (Qiagen or Invitrogen) according to the manufacturers' instructions. Subtype specific RT-PCR assays using specific primers for M, NP, HA, and NA genes were used for subtype identification or confirmation (12-14).

**Table 1.** Wild birds sampled in different provinces of Iran before start of H5Nx outbreaks (2014- 2015).

Family name	Bird name	Scientific name	Number of sampled birds/province	Total number of sampled birds
	Pochard	Aythya ferina	1 <sup>M</sup> +1 <sup>GO</sup>	2
	White Fronted Geese	Anser albifrons	4 <sup>M</sup>	4
	Tufted Duck	Aythya fuligula	1 <sup>M</sup>	1
	Ruddy Shelduck	Tadorna ferruginea	3 <sup>M</sup> +1 <sup>GO</sup> +1 <sup>WA</sup> +1 <sup>G</sup>	6
	Eurasian Teal	Anas crecca	2 <sup>M</sup> +1 <sup>GO</sup> +2 <sup>WA</sup> +2 <sup>Q</sup> +20 <sup>G</sup>	27
	Graylag Goose	Anser anser	1 <sup>M</sup> +1 <sup>G</sup>	2
	Mute Swan	Cygnus olor	1 <sup>M</sup>	1
	Mallard	Anas platyrhynchos	6 <sup>M</sup> +1 <sup>GO</sup>	7
Anatidae	Lesser White Fronted Geese	Anser erythropus	1 <sup>M</sup>	1
	Black Swan	Cygnus atratus	1 <sup>M</sup> +2 <sup>Q</sup> +1 <sup>G</sup>	4
	Ferruginous Duck	Aythya nyroca	1 <sup>M</sup> +2 <sup>G</sup>	3
	Shelduck	Tadorna tadorna	1 <sup>G</sup>	1
	Northern Pintail	Anas acuta	1 <sup>GO</sup>	1
	Whooper Swan	Cygnus cygnus	1 <sup>Q</sup> +1 <sup>G</sup>	2
	Common Goldeneye	Bucephala clangula	1 <sup>GO</sup>	1
	Common Merganser	Mergus merganser	1 <sup>GO</sup>	1
	Bean Goose	Anser fabalis	2 <sup>G</sup>	2
	Mandarin	Aix galericulata	1 <sup>Q</sup>	1
Gruidae	Demoiselle Crane	Anthropoides virgo	1 <sup>M</sup>	1
Phoenicopteridae	Flamingo	Phoenicopterus roseus	1 <sup>Go</sup>	1
	Common Kestrel	Falco tinnunculus	4 <sup>GO</sup>	4
	Common Buzzard	Buteo buteo	13 <sup>GO</sup>	13
	Egyptian Vulture	Neophron percnopterus	3 <sup>GO</sup>	3
	Marsh Harrier	Circus aeruginosus	2 <sup>GO</sup>	2
	Black Kite	Milvus migrans	1 <sup>GO</sup>	1
Accipitridae	Eastern Imperial Eagle	Aquila heliaca	1 <sup>GO</sup>	1
	Sea Eagle	Haliaeetus leucocephalus	1 <sup>GO</sup>	1
	Greater Spotted Eagle	Clanga clanga	3 <sup>GO</sup>	3
	Montagu's Harrier	Circus pygargus	1 <sup>GO</sup>	1
	Long-legged Buzzard	Buteo rufinus	1 <sup>Go</sup>	1
Phasianidae	Peafowl	Pavo muticus	1 <sup>GO</sup> +1 <sup>G</sup> +1 <sup>Q</sup>	3
	Pheasant	Phasianus colchicus	1 <sup>Go</sup> +4 <sup>M</sup> +1 <sup>Q</sup>	6
Numididae	Guinea Fowl	Numida meleagris	1 <sup>Go</sup>	1
Strigidae	Tawny Owl	Strix aluco	4 <sup>GO</sup>	4
	Eurasian Eagle-Owl	Bubo bubo	1 <sup>GO</sup>	1
	Unidentified Species			20
				134

Abbreviations: G: Gilan, GO: Gorgan, Q: Qom, M: Mazandaran, WA: West Azerbaijan

Table 2. Wild birds sampled in different provinces of Iran during H5Nx outbreaks (Sep. 2016- Feb 2018).

Family name	Bird name	Scientific name	Total number of sampled birds	Number of sampled birds/province	Pos/Neg H5Nx HPAIV results	Type
	Mute Swan	Cygnus olor	2 <sup>Q</sup> +1 <sup>G</sup> +1 <sup>GO</sup> +2 <sup>G</sup> +1 <sup>T</sup> *	7	+/-/-/+	H <sub>5</sub> N <sub>8</sub> /H <sub>5</sub> N <sub>6</sub> *
	Black Swan	Cygnus aratus	1 <sup>T</sup>	1	+	H <sub>5</sub> N <sub>8</sub>
	Whooper Swan	Cygnus	4 <sup>G</sup> +4 <sup>G</sup> +10 <sup>M</sup> +4 <sup>M</sup>	22	+/-/+/-	H <sub>5</sub> N <sub>8</sub>
	Greyleg Goose	Anser anser	4 <sup>MR</sup> +2 <sup>M</sup> +1 <sup>Q</sup> +1 <sup>KO</sup>	8	+/-/-/-	H <sub>5</sub> N <sub>8</sub>
	Eurasian Wigeon	Anas Penelope	1 <sup>KR</sup>	1	-	-
	Ruddy Shelduck	Tadorna ferruginea	8 <sup>EA</sup> +1 <sup>G</sup>	9	+/+	H <sub>5</sub> N <sub>1</sub> /H <sub>5</sub> N <sub>6</sub>
	Gadwall	Anas strepera	1 <sup>KU</sup> +18 <sup>G</sup>	19	-/+	H <sub>5</sub> N <sub>6</sub>
	Common Teal	Anas Crecca	19 <sup>EA</sup> +1 <sup>M</sup> +1 <sup>G</sup> +1 <sup>MR</sup>	22	+/-/+/-	H <sub>5</sub> N <sub>1</sub> /H <sub>5</sub> N <sub>8</sub>
Anatidae	Mallard	Anas platyrhynchos	13 <sup>EA</sup> +4 <sup>KE</sup> +7 <sup>GL</sup> +1 <sup>MR</sup> +1 <sup>M</sup>	26	+/-/-/-/+	H <sub>5</sub> N <sub>1</sub> /H <sub>5</sub> N <sub>8</sub>
	Shoveler	Anas clypeata	1 <sup>G</sup>	1	+	H <sub>5</sub> N <sub>6</sub>
	Common Pochard	Aythya ferina	4 <sup>M</sup>	4	-	-
	Pochard Ferruginous	Aythya nyroca	1 <sup>MR</sup>	1	-	-
	Tufted Duck	Aythya fuligula	1 <sup>M</sup>	1	+	H <sub>5</sub> N <sub>8</sub>
	Red-Crested Pochard	Netta rufina	1 <sup>M</sup>	1	+	H <sub>5</sub> N <sub>8</sub>
	Unidentified duck sp.	??	4 <sup>M</sup> +4 <sup>T</sup>	8	-	-
	Domestic Anatidae	Anas sp.	8 <sup>L</sup> +6 <sup>T</sup>	14	+/+	H <sub>5</sub> N <sub>8</sub> /H <sub>5</sub> N <sub>8</sub>
	Common Buzzard (Steppe Buzzard)	Bueto bueto	1 <sup>CH</sup> +3 <sup>IS</sup>	4	+/+	H <sub>5</sub> N <sub>8</sub>
Accipiteridae	Golden Eagle	Aquila Chrysaetos	2 <sup>EA</sup> + 1 <sup>IL</sup>	3	+/-	H <sub>5</sub> N <sub>1</sub>
	Night Heron	Nycticorax	1 <sup>KU</sup>	1	-	-
Ardeidae	Bittern	Botaurus Stellaris	2 <sup>KU</sup> +1 <sup>MR</sup>	3	-/+	H <sub>5</sub> N <sub>8</sub>
	Little Egret	Egretta garzetta	3 <sup>IS</sup>	3	+	H <sub>5</sub> N <sub>8</sub>
Charadriidae	Northern Lapwing)	Vanellus vanellus	1 <sup>M</sup>	1	-	-
Ciconiidae	White Stork	Ciconia ciconia	1 <sup>KU</sup>	1	+	H <sub>5</sub> N <sub>8</sub>
Cordaciidae	Roller	Coracias garrulus	1 <sup>KR</sup>	1	-	--
	Carrion Crow	Corvus corone	1 <sup>MR</sup> +35 <sup>IS</sup> +5 <sup>CH</sup>	41	-/+	H <sub>5</sub> N <sub>8</sub>
Corvidae	Magpie	Pica	1 <sup>KR</sup> +2 <sup>Q</sup>	3	-/+	- / H <sub>5</sub> N <sub>x</sub>
	Common Rook	Corvus frugilegus	5 <sup>KR</sup> +2 <sup>Q</sup>	7	-/+	- / H <sub>5</sub> N <sub>x</sub>
	Rock Dove (Rock Pigeon)	Columba livia	1 <sup>MR</sup> +5 <sup>M</sup> +1 <sup>IL</sup>	7	-/-	-
Columbidae	Common Wood Pigeon	Columba palumbus	10 <sup>KR</sup>	10	-	-
	Laughing Dove	Spilopelia senegalensis	14 <sup>H</sup>	14	-	-
	Eurasian Collared Dove	Streptopelia decaocta	1 <sup>IL</sup>	1	-	-
	Common Gull (Mew Gull)	Larus canus	10 <sup>EA</sup> +1 <sup>MR</sup>	11	+/+	H <sub>5</sub> N <sub>8</sub> / H <sub>5</sub> N <sub>1</sub>
Laridae	Black-headed Gull	Larus ridibunds	1 <sup>G</sup> +1 <sup>M</sup> +8 <sup>T</sup>	10	-/-/+	H <sub>5</sub> N <sub>8</sub>
	Great Black-headed Gull	Larus Ichthyaeus	4 <sup>G</sup>	4	+	H <sub>5</sub> N <sub>6</sub>
Otididae	Little Bustard	Tetrax tetrax	1 <sup>M</sup>	1	-	-
Otidiforms	Bustard	Chlamydotis macqueeni	2 <sup>Q</sup>	2	+	H <sub>5</sub> N <sub>2</sub>
Passeridae	House Sparrow	Passer Dpmeticus	8 <sup>IS</sup>	8	+	H <sub>5</sub> N <sub>8</sub>
Pelecanidae	Dolmatian Pelican	Pelcanus crispus	2 <sup>G</sup> +2 <sup>GO</sup> +1 <sup>AR</sup> +1 <sup>G</sup> *	6	+/-/+	H <sub>5</sub> N <sub>8</sub> /H <sub>5</sub> N <sub>6</sub> *
	Common Pheasant	Phasianus colchicus	1 <sup>M</sup>	1	-	-
Phasianidae	Chukar	Alectoris Chukar	2 <sup>MR</sup>	2	-	-
	Peafowl	Pavo Cristatus	1 <sup>MR</sup> +16 <sup>QZ</sup>	17	-/+	H <sub>5</sub> N <sub>2</sub>
		Dendrocopos syriacus	1 <sup>M</sup>	1	-	-
Picidae	Syrian Woodpecker	(Picoides syr Iacus)				
Phalacrocoracidae	Cormmorant	Phalacrocorax carbo	3 <sup>MR</sup> +1 <sup>IL</sup> +4 <sup>QZ</sup>	10	-/-	-
Phoenicopteridae	Greater Flamingo	Phoenicopterus ruber	1 <sup>MR</sup> +1 <sup>MR</sup> +1 <sup>G</sup>	3	-/+	H <sub>5</sub> N <sub>8</sub> /H <sub>5</sub> N <sub>6</sub>
Podicipedidae	Great Crested Grebe	Podiceps cristatus	5 <sup>M</sup> +2 <sup>KU</sup>	7	+/-	H <sub>5</sub> N <sub>8</sub>
Recurvirostridae	Black-winged Stilt	Himantopus himantopus	6 <sup>EA</sup>	6	+	H <sub>5</sub> N <sub>1</sub>
Rallidae	Eurasian Coot	Fulica atra	3 <sup>KU</sup>	3	-	-
Scolopacidae	Common Sandpiper	Actitis hypoleucos (Tringa hypoleucos)	1 <sup>KE</sup>	1	-	-
Unidentified Wild Birds			176 <sup>KH</sup> +1 <sup>Y</sup> +100 <sup>GL</sup>	277	-	-

**Abbreviations.** AR: Ardabil, CH: Charmahal and Bakhtiari, EA: East Azerbaijan, G: Gilan, GL: Golestan, H: Hormozgan, IL: Ilam, IS: Isfahan, KH: Khorasan Razavi, KR: Kermanshah, KU: Kurdistan, L: Lorestan, M: Mazandaran, MR: Markazi, Q: Qom, QZ: Qazvin, T: Tehran, Y: Yazd.

**Table 3.** Wild birds sampled in different provinces of Iran after H5Nx outbreaks (2018- 2019).

Family name	Bird name	Scientific name	Number of sampled birds/province	Total number of sampled birds
Anatidae	Mallard	Anas platyrhynchos	1 <sup>T</sup> +1 <sup>KU</sup>	2
Laridae	Unidentified Gull sp.	Larus sp.	1 <sup>H</sup> +4 <sup>S</sup> +2 <sup>EA</sup>	7
	Great Egret	Egretta alba	1 <sup>S</sup> +7 <sup>M</sup>	8
Ardeidae	Grey Heron	Ardea cinerea	1 <sup>H</sup> +2 <sup>EA</sup> +1 <sup>T</sup> +1 <sup>A</sup>	5
Rallidae	Eurasian Coot	Fulica atra	2 <sup>KR</sup>	2
Phalacrocoracidae	Great Cormorant	Phalacrocorax carbo	1 <sup>Z</sup>	1
Phoenicopteridae	Greater Flamingo	Phoenicopterus ruber	30 <sup>F.2</sup>	30
	Common Wood Pigeon	<i>Columba palumbus</i>	32 <sup>KR</sup> +2 <sup>Lo</sup>	34
Columbidae	Laughing Dove	<i>Spilopelia senegalensis</i>	5 <sup>S</sup>	5
Pelecanidae	Dalmation Pelican	Pelecanus crispus	3 <sup>H</sup> +1 <sup>G</sup> +3 <sup>Q</sup>	7
Phasianidae	Partridge		2 <sup>Z</sup> +1 <sup>Y</sup>	3
Gruidae	Common Crane		1 <sup>MR</sup>	1
	Step Eagle		33 <sup>F.1</sup>	33
Accipitridae	Golden Eagle	<i>Aquila chrysaetos</i>	3 <sup>Q</sup>	3
	Steppe Buzzard	<i>Buteo vulpinus</i>	3 <sup>Q</sup>	3
Falconidae	Common Kestrel	<i>Falco tinnunculus</i>	3 <sup>Q</sup>	3
Sturnidae	Starling		10 <sup>MR</sup>	10
				157

Abbreviations: A: Alborz, EA: East Azerbaijan, F: Fars, G: Gilan, H: Hormozgan, KR: Kermanshah, KU: Kurdistan, M: Mazandaran, MR: Markazi, Q: Qom, S: Sistan, T: Tehran, WA: West Azerbaijan, Y: Yazd, Z: Zanjan  
1: Mortality due to intoxication, 2: Thunderbolt accident



**Figure 1.** Geographical distribution of sampling sites in Iran. Blue spots indicate negative samples and red spots indicate positive samples (individual or outbreak cases). Map generated using Google Maps (interactive map available at <https://www.google.com/maps/d/viewer?mid=1AK4RKq3-LHWCmY-9NsadWx7Pcu2SdT0k&ll>).

### 3. Results

#### 3.1. Detection of HPAIV in Iran

##### 3.1.1. H5Nx HPAIV Surveillance in 2014-2016 (Pre-Epidemic Period)

The pre-epidemic surveillance study in wild birds in five provinces revealed no H5Nx HPAIV positive birds. In addition, passive surveillance of dead wild birds and birds kept in zoological gardens, zoos, or rehabilitation centers in other provinces of Iran did not result in any positive H5 cases.

##### 3.2. AIV Outbreaks Detected During the Surveillance Period

###### 3.2.1. H5N1 Outbreak (Sep 2016)

In September 2016, wild-bird mortality was reported in Bonab city (West Azerbaijan province) close to Urmia Lake and Gharegheshlagh Wetland.

Approximately 300-400 dead birds, mainly ducks, died during the outbreak. Other bird species including gull spp., Eurasian teals, ruddy shelducks, stilts, mallards, and eagles were also found dead. During the field investigation, 10 gulls, 19 Eurasian teals, 8 ruddy shelducks, 6 stilts, 13 mallards, and 2 eagles were collected from the field. The H5N1 subtype was identified in several dead birds. H5N1 outbreaks were also consequently reported from poultry farms and outdoor poultry with high morbidity and mortality (personal communication).

###### 3.2.2. H5N6 Outbreak (Nov 2016)

In November 2016, the first die-off of wild birds in several wetlands southeast of the Caspian Sea was reported. The first reported foci of the epidemic were in

Miankaleh wetland, a major wintering site of many species of migratory birds. Great Crested Grebe mergansers (*Podiceps cristatus*) and tufted ducks (*Aythya fuligula*) were the main bird species reported dead. Due to the large size of the wetland, an accurate estimation of the number of dead birds was not possible. However, the approximate estimation indicated that hundreds of mergansers and grebes died during this outbreak. In postmortem examinations, extensive hemorrhage in internal organs, including the liver, lungs, spleen, and intestines, was evident. During the field investigation, hundreds of crows were observed feeding on the dead birds; however no dead crows were observed or reported in this area. H5N6 was identified in samples collected from four dead birds.

### 3.2.3. H5N8 Outbreak (Dec 2016 – Feb 2017)

In almost the same period, approximately 30 whooper swans (*Cygnus cygnus*) in three small wetlands (Sorkhroud, Fereydonkenar, and Azbaran) were reported approximately 100 km away from the H5N6 outbreak. Interestingly, mortality was exceptionally low in swans during this outbreak, as only 30 out of approximately 10,000 wintering mute and whooper swans died due to the infection. Two mute swans (*Cygnus olor*) were also found dead in Qom province, and the H5N8 subtype was identified as the cause. Consequently, the virus was also found in the Miankaleh wetland.

In December 2016, a new devastating wild bird die-off in the center of Iran was reported. In Mighan wetland (Markazi province) several different bird species, especially graylag geese (*Anser anser*), were reported dead. Among the sick birds, nervous symptoms were prominently observed. Approximately 3000 wild birds died in the Mighan wetland, and graylag geese constituted approximately 90% of the mortality. Other dead species included, 167 mallards (*Anas platyrhynchos*), 69 Eurasian coots (*Fulica atra*), 67 common cranes (*Grus grus*), 30 common redshanks (*Tringa totanus*), 23 greater flamingos (*Phoenicopterus roseus*), 21 gulls (unidentified species), 18 Shelducks

(*Tadorna tadorna*), 13 ruddy shelducks (*Tadorna ferruginea*), 10 little cormorants (*Microcarbo niger*), and Eurasian bitterns (*Botaurus stellaris*). Nearly the whole graylag goose population in this wetland was wiped out by the outbreak. The dead birds were found in different areas of the 250 square kilometer wetland. A new subtype of the HPAIV, i.e. H5N8, was identified and characterized from this outbreak.

New outbreaks or individual cases were further reported from several wetlands southwest of the Caspian Sea, such as Anzali and Ardabil; wild birds such as Dalmation pelicans (*Pelecanus crispus*), whooper swans, and Eurasian teals were found dead and consequently tested positive for H5N8 HPAIV.

H5N8 outbreaks were also reported in poultry farms and caused huge economic losses. In several central and southern provinces of Iran, especially areas with high numbers of small poultry farms such as Shahrekord (Chahar Mahaal and Bakhtiari province) and Qom (Qom province), individual wild birds, mainly scavengers, birds of prey, and crows, were reported dead. Although several wetlands with high Anatidae populations exist in those provinces, and despite extensive surveillance of the area, no cases of mortality were reported in those wetlands during the same period. Those mortalities may have happened because of the inappropriate disposal of domestic poultry carcasses and the scavenging of HPAIV-contaminated carcasses by wild birds.

The last reported cases in this season occurred in March 2017, when several positive whooper and mute swans were found in Saei Park, a public park with small ponds in the center of Tehran.

No more positive cases were reported in wild birds between March and September 2017, although surveillance plans in each province were intensified. However, H5N8 outbreaks in the poultry sector continued.

### 3.2.4. Second H5N8 Outbreak (Sep-Dec 2017)

From September 2017 to January 2018, three limited outbreaks occurred in Chitgar pond, a large artificial pond close to Tehran. More than 100 (out of

approximately 5000) black-headed gulls (*Larus ridibundus*) were found dead and consequently found to be positive for H5N8 HPAIV.

### 3.2.5. Second H5N6 Outbreak (Feb 2018)

In February 2018, a mass die-off among wild birds in Bojaq National Park, located close to the Caspian Sea, was reported by park rangers (Figure 1). Within two weeks, from 20,000 wild birds of different species that were counted three weeks before the outbreak in this park, approximately 4000, mainly gadwalls (*Anas strepera*) were reported dead. Assuming that no migration occurred, approximately 20% of the local population of gadwalls might have perished during that outbreak.

### 3.2.6. HPAIV H5Nx Surveillance in 2018-2019 (Post-Epidemic Period) (March 2018 – June 2019)

During the post-epidemic period (until July 2019), no new case of HPAIV-H5 infection was reported, and analyses of 160 dead wild birds did not reveal any positive H5 results.

## 3.3. Temporal and Geographical Distribution of HPAIV

Within five years of HPAIV surveillance in different provinces, several extensive outbreaks, and a high number of individual positive dead birds were identified in different parts of the country (Figure 1). The number of samples collected in this study was not distributed evenly over different geographical regions or time zones, mainly due to logistic issues.

Interestingly, all samples collected before the introduction of HPAIVs and thereafter tested negative in the current study. However, within the geographical and time ranges of the outbreaks, even individual positive wild birds were found. Three major outbreaks were reported from the wetlands south of the Caspian Sea: one close to Lake Urmia, one in an artificial pond close to Tehran, and a third major outbreak in the center of Iran.

## 4. Discussion

In recent years, H5Nx HPAI viruses (H5N1, H5N2, H5N6, and H5N8) have been increasingly detected in

domestic and wild birds in Asian, European, and African countries and has caused high mortality in wild bird populations and economic losses in poultry (15-17). The co-circulation of LPAIVs (especially H9N2) and different subtypes and lineages of HPAIVs (H5N1 2.3.2.1c, H5N8 2.3.4.4a, H5N8 2.3.4.4b, H5N6 2.3.4.4c) in poultry with frequent spill-over transmissions into wild bird populations has been reported frequently in the last decade. Because of expansion and species-barrier cross-over of HPAIVs H5Nx, the influenza infections are no longer restricted to poultry. Indeed, the viruses causing the outbreaks may efficiently circulate in wild bird populations and cause unlimited expansion through migratory flyways.

Iran is located on the southern shore of the Caspian Sea, and its wetlands represent major wintering and stopover sites for several million migratory birds from Siberia and northern Russia. Those migratory birds usually arrive in October and either remain until February/March or migrate further south (10). The high potential for the introduction of highly pathogenic influenza viruses into the countries located in the migratory flyways has already been shown (6). Obviously, after the initial introduction of such pathogens, further expansion of the outbreaks relies mainly on the local transport of live poultry and poultry meat and by-products.

Because Iran is located on the path of two major migratory flyways, wild birds may play a major role in the introduction of HPAIVs into the country. After the emergence of several new subtypes of H5Nx HPAI viruses, the establishment of a surveillance study to monitor any introduction of HPAIVs into the wild bird populations in Iran required a high priority. The important role of waterbirds, especially waterfowl, as a reservoir for avian influenza viruses of all subtypes is well known from intensive investigations from many regions of the world (18, 19). The monitoring of avian influenza among wild birds in natural habitats and in areas at risk of transmission between domestic poultry and wild birds will increase the knowledge of

epidemiology, ecology, and genetic relationships of AIV infections. This knowledge will facilitate risk assessments concerning poultry and wild bird populations and provide information on currently circulating AIV, which might also have the potential to become important for human health (10).

In 2016-2017, the HPAIV H5N8 strain spread worldwide, and many countries reported that the occurrence of the virus caused symptoms in both poultry and wild birds. For example, 29 European countries were affected. In Germany alone, within 7 months (Nov. 2016 - May 2017) more than 1,150 cases of HPAI H5N8 in wild birds and 107 outbreaks in birds kept in captivity (92 poultry holdings and 15 zoos/animal parks) were reported (7). In Iran, the first evidence for the introduction of H5N8 viruses was found in December 2016, and then major outbreaks were reported in several wetlands in the north of the country. The most devastating one occurred in the center of Iran in the Mighan wetland and caused high mortality (> 3000) in the graylag geese population. The viruses were most probably introduced initially by migratory birds, sparking an epidemic among wild birds with occasional incursions into poultry holdings. However, further expansion into commercial poultry holdings most probably occurred through the transport of poultry products and by-products.

In addition to HPAI H5N8 clade 2.3.4.4b outbreaks, two outbreaks of H5N6 were reported during this surveillance study. The first outbreak with limited mortality occurred in 2016, and the second one was registered in February 2018 and caused a high level of mortality, especially among gadwalls, although it also infected other wild bird species.

In total, five major outbreaks and many individual positive cases were identified. The geographical distribution of positive samples revealed further significant differences. However, with respect to the size of the country and the wetlands, missing individual HPAIV positive wild birds during the surveillance study cannot be excluded. The novel HPAIV H5N8 clade 2.3.4.4B was reported as a potential risk by the

FAO in September 2016 when it was detected in migratory birds in the Tyva Republic, Russian Federation (20). In October, Hungary and Poland reported the first cases of HPAI H5N8 clade 2.3.4.4b in dead wild birds. It may be concluded that the virus logically should have arrived in the wetlands of Iran between September and October 2016; however, the first outbreak in Iran was noted in November 2016. Therefore, some individual dead birds may not have been detected until a notable number of mortalities had occurred. The virus was consequently transferred into poultry farms and caused mortality and economic loss (6, 21). Therefore, protecting domestic poultry holdings from close contact with wild birds by creating physical and functional barriers has the highest priority.

Wild birds are the victims of the HPAIV emergence in South-East Asia due to the mismanagement of poultry farms and live bird markets. Intra- and intercontinental transmission of HPAIVs through migratory wild birds is an unavoidable consequence of the anthropogenic facilitation of HPAIVs emergence; in many cases, substantial gaps in the biosecurity of poultry farms such as the potential contact with wild birds, mode and frequency of farm visits, level of biosecurity, and density of poultry holdings in a specific region may have eased the path for virus entry and further expansion of the outbreak (7, 22).

#### **Authors' Contribution**

Study concept and design: S. M. M. and S. F.

Acquisition of data: S. M. M.

Analysis and interpretation of data: S. M. M. and S. F.

Drafting of the manuscript: S. M. M. and S. F.

Critical revision of the manuscript for important intellectual content: S. F. and H. M.

Statistical analysis: S. F. and M. V.

Administrative, technical, and material support: S. M. M., N. Sh. and Gh. N. B

#### **Ethics**

All the procedures and animal handling were approved by the Animal Ethics committee at the Azad

University of Tehran, Iran under the project number of 2019-1457412-2.

### Conflict of Interest

The authors declare that they have no conflict of interest.

### Grant Support

This article was extracted from PhD thesis in Avian Diseases entitled Study of Ecology and Epidemiology of Avian Influenza Viruses Type A by molecular methods. This research was not financially supported by any organizations.

### Acknowledgment

The authors would like to appreciate all rangers of department of environment (DOE), vets of DOE and IVO( Iran Veterinarian organization) and wildlife diseases lab in Razi institution.

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