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Epidemiological monitoring of avian influenza in the Republic of Crimea in 2019–2020

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SUMMARY

The paper presents results of avian influenza epidemiological monitoring in the Republic of Crimea in 2019–2020. The attention was focused on the study of water basins of the Azov and Black Seas, the Sivash Lagoon and freshwater lakes in the Feodosia Urban Okrug, Leninsky, Sovetsky, Nizhnegorsky, Chernomorsky and Saksky Raions to detect the avian influenza virus circulation. Examination of the above mentioned areas showed that some freshwater reservoirs became shallow and dry, and aquatic vegetation degraded. The natural biotope analysis conducted in 2019 and 2020 showed a decreased number of semiaquatic wild birds. The pathological material was sampled from semiaquatic and migratory wild birds, as well as from poultry kept in poultry farms and backyards. The collected samples were tested using real-time RT-PCR. In 2019, the AIV type A (H9) genome was detected in one fecal sample taken from wild birds near Kuchuk-Adzhigol Lake in Feodosia Urban Okrug. The AIV type A (H5) genome was detected in 2020 during laboratory testing of pathological material taken from the remains of a mute swan within the shoreline of a freshwater lake near the Ermakovo settlement of the Dzhankoysky Raion. The genetic analysis was performed in the FGBI "ARRIAH" (Vladimir), and the N8 subtype neuraminidase of the influenza virus isolate was determined. The comparative genetic analysis of 258 bp nucleic acid sequences of the AIV H gene fragment showed that the identified isolate belongs to the Asian genetic lineage of highly pathogenic AIV subtype H5 (clade 2.3.4.4) associated with the epidemic spread in Asia, Europe, the Middle East and Africa in 2016–2020.

Keywords: wild migratory birds, semiaquatic birds, avian influenza, migration, bird fauna, epidemiological monitoring

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Эпизоотологический мониторинг гриппа птиц на территории Республики Крым в 2019–2020 гг.

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РЕЗЮМЕ

В статье представлены результаты эпизоотологического мониторинга гриппа птиц на территории Республики Крым в 2019–2020 гг. При изучении циркуляции вируса гриппа птиц особое внимание уделено акваториям Азовского и Черного морей, заливу Сиваш и пресноводным озерам городского округа Феодосия, Ленинского, Советского, Нижнегорского, Черноморского и Сакского районов. При обследовании вышеуказанных районов было отмечено обмеление и пересыхание некоторых пресноводных водоемов, деградация водной растительности. Анализ состояния обследуемых природных биотопов за 2019 и 2020 гг. показал снижение численности диких околоводных птиц. Произведен отбор проб патологического материала от околоводной и дикой перелетной птицы, а также от птиц, содержащихся на птицефабриках и в личных подсобных хозяйствах граждан. Полученные образцы исследовали методом полимеразной цепной реакции в реальном времени с обратной транскрипцией. В 2019 г. в одной пробе помета от дикой птицы с озера Кучук-Аджиголь городского округа Феодосия обнаружен геном вируса гриппа А птиц подтипа H9. В результате проведенных в 2020 г. лабораторных исследований патологического материала (останки лебедя-шипуна), отобранного на береговой линии пресноводного озера вблизи с. Ермаково Джанкойского района, выявлен геном вируса гриппа А подтипа H5. На базе ФГБУ «ВНИИЗЖ» (г. Владимир) был проведен генетический анализ и определен подтип нейраминидазы выделенного изолята вируса гриппа – N8. Сравнительный генетический анализ нуклеиновых последовательностей фрагмента гена H длиной 258 н. о. вируса гриппа птиц показал, что выявленный изолят принадлежит к азиатской генетической линии вируса высокопатогенного гриппа птиц подтипа H5 (клада 2.3.4.4), получившего эпизоотическое распространение в 2016–2020 гг. в странах Азии, Европы, Ближнего Востока и Африки.

Ключевые слова: дикая перелетная птица, околоводная птица, грипп птиц, миграция, орнитофауна, эпизоотологический мониторинг

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INTRODUCTION

Avian influenza (AI) is a highly contagious viral disease of birds, subject to the World Organisation for Animal Health (OIE) notification. It causes enormous economic damage and poses a serious threat to poultry farms around the globe. The causative agent is an RNA virus belonging to the family *Orthomyxoviridae*, type A (*Influenza A*). There are 16 known hemagglutinin antigens (H1–H16) and 9 neuraminidase antigens (N1–N9). The AI agent has unique abilities to change its antigenic structure and cross the species barrier [1–8].

Avian influenza virus (AIV) is steadily circulating in wild migratory bird populations in many countries. Aquatic and semiaquatic birds are the primary natural reservoir of the virus [1, 2, 7–9]. The Republic of Crimea located at the crossroads of wild bird migration routes from Asia to European and African countries and back, is a stopover and wintering place for many wild semiaquatic bird species. It was primarily the AIV subtype H5N1 known to be circulating until 2014 and causing epidemics in the Crimea in 2005, 2006 and 2008 [5, 10, 11]. At present the AIV subtype H5N8 has become globally widespread and was detected in domestic and wild bird populations in Europe, Asia and Africa. In the Russian Federation the highly pathogenic H5N8 influenza virus was initially isolated from dead wild birds at Uvs Nuur Lake (Republic of Tyva) in May 2016 [2, 6–8, 9, 11–15]. Then the disease became widespread and was recorded in both wild and domestic birds in the immediate vicinity of the Crimean Peninsula, namely in the Rostov Oblast, Krasnodar Krai, as well as in the Kherson and Odessa Oblasts in Ukraine [3, 16]. The next epizootic wave started in August 2020 in the Southern Urals and Western Siberia and affected most of the European countries by the end of the year. The current epidemic is caused by H5N8, H5N5, H5N4, H5N3, H5N1 subtype viruses belonging to genetic clade 2.3.4.4, which originates from the Middle East [6–8].

From 2009 to the present time no AI disease and mortality cases have been reported in backyard and commercial poultry on the territory of Crimea. But taking into account that the AIV (H5N8) genome was detected in a mute swan in the territory of the peninsula in 2017, and the animal products imported from the Penza Oblast [11, 16] tested positive for AI (subtype H9) in 2018, there remains a real threat of AIV introduction into backyard and commercial flocks in the Republic of Crimea.

Thus, the AI epidemiological monitoring is of relevant importance on the territory of the Republic of Crimea.

MATERIALS AND METHODS

The tests were conducted on the basis of the Laboratory for Molecular Diagnostics of the Laboratory and Diagnostic Center of the FGBI "ARRIAH" Branch in the Republic of Crimea in 2019–2020.

Biological material was collected from wild birds during expeditions to the coast of the Azov and Black Seas, the Sivash Lagoon and freshwater lakes in the Feodosia Urban Okrug, Leninsky, Sovetsky, Nizhnegorsky, Chernomorsky and Saki Raions with participation of the Rosselkhoznadzor Administration for the Republic of Crimea and Sevastopol City (Rosselkhoznadzor) specialists. The expeditions were arranged pursuant to the research work plan of the FGBI "ARRIAH" Branch in the Republic of Crimea and state epidemiological monitoring plans.

The species identification of wild birds subjected to biomaterial sampling was carried out using a bird field guide [17]. During the expeditions the state of natural biotopes was assessed and the number of bird fauna in this territory was calculated.

The material was delivered to the laboratory in sealed water-proof insulated containers with cool packs in compliance with current regulations. Laboratory tests were carried out using Russia-manufactured commercial test kits in accordance with the Veterinary Rules for Avian Influenza A Laboratory Diagnosis [18].

Biomaterial samples from wild birds shot in various hunting areas of the Crimea, samples from poultry kept in poultry farms and backyards, as well as samples of animal products were sent to the laboratory of the FGBI "ARRIAH" Branch in the Republic of Crimea.

Identification of the AIV genome was carried out by extraction of total nucleic acid from pathological material, reverse transcription of the obtained RNA and amplification of specific sites of the cDNA derived from the influenza A virus using real-time polymerase chain reaction (real time RT-PCR). The following was used: a GRIPP commercial test

Table 1
Pathological material sampling from wild birds in 2019

Sampling date	Type of pathological material	Number of samples	Sampling site
February 18, 2019	Faeces (wild birds)	1	6.5 km from Znamenskoye settlement (Spit Belyaus), Lake Donuzlav shore (45.346111, 32.954625)
	Eurasian coot (*Fulica atra*), Gull (*Larus*) (carcasses)	4	
	Faeces (wild birds)	1	Sasyk-Sivash Lake, Yevpatoria (45.200887, 33.415658)
	Wild birds (carcasses)	9	Panskoe Lake, Chernomorsky Raion
August 01, 2019	Faeces (wild birds)	5	3 km from Yermakovo settlement, Dzhankoysky Raion
	Gull (*Larus*) (remains)	1	
	Faeces (wild birds)	25	5 km from Yermakovo settlement, Dzhankoysky Raion, shore of Sivash Lagoon
	Faeces (wild birds)	3	5 km from Tomashevka settlement, Dzhankoysky Raion, Aigulskoye Lake
	Faeces (wild birds)	8	3 km east of Tselinnoye settlement, Dzhankoysky Raion
	Faeces (wild birds)	10	3 km from Melkovodnoye settlement, Dzhankoysky Raion, Aigulskoye Lake
August 08, 2019	Faeces (wild birds)	1	area of the Ablyamitsky bridge, Lake Donuzlav
	Faeces (wild birds)	12	5 km from Znamenskoye settlement, Lake Donuzlav
	Faeces (wild birds)	6	5 km from Mezhvodnoye settlement, Lake Panskoye, Chernomorsky Raion
	Faeces (wild birds)	7	5 km from Yevpatoria, Sasyk-Sivash Lake
	Sparrow (*Passeridae*) (carcass)	1	
	Gull (*Larus*) (remains)	1	
October 18, 2019	Mute swan (*Cygnus olor*) (remains)	1	Sasyk-Sivash Lake (45.204513, 33.410529)
	Mute swan (*Cygnus olor*) (remains)	1	Sasyk-Sivash Lake (45.204128, 33.411204)
	Faeces (wild birds)	4	Sasyk-Sivash Lake
	Faeces (wild birds)	18	shore of Lake Donuzlav, Ablyamitsky bridge
	Faeces (wild birds)	15	shore of Lake Donuzlav, Spit Belyaus, 5 km from Znamenskoye settlement
November 25, 2019	Faeces (wild birds)	27	Lake Kuchuk-Ajigol, Feodosia Urban Okrug
	Total	161	

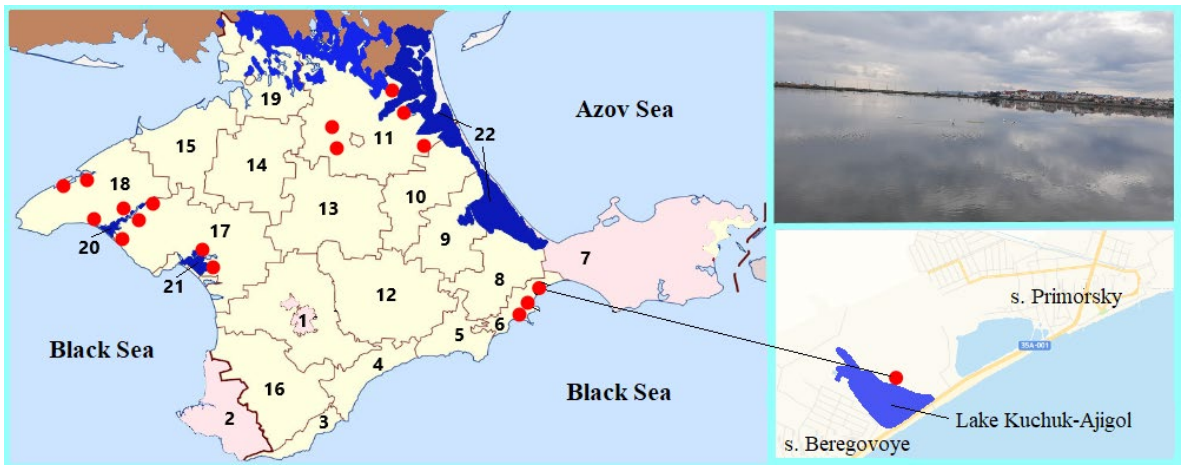

Fig. 1. Sampling of pathological and biological material from semiaquatic wild birds in 2019

● – location of sites for pathological and biological material sampling from wild birds

1. Simferopol
2. Sevastopol
3. Yalta
4. Alushta
5. Sudak
6. Feodosia
7. Leninsky Raion
8. Kirovsky Raion
9. Sovetsky Raion
10. Nizhnegorsky Raion
11. Dzhankoysky Raion
12. Belgorodsky Raion
13. Krasnogvardeysky Raion
14. Pervomaysky Raion
15. Razdolnensky Raion
16. Bakhchisaraysky Raion
17. Saksky Raion
18. Chernomorsky Raion
19. Krasnoperekopsky Raion
20. Donuzlav Lake
21. Sasyk-Sivash Lake
22. Sivash Lagoon

kit (the Rospotrebnadzor Central Research Institute of Epidemiology, Russia) for PCR detection and differentiation of influenza virus (*Influenza A virus*) and differentiation of subtypes (A H5, H7, H9), which includes AmpliPrime RIBO-Sorb reagents for RNA extraction, Reverta-L for the reverse transcription and reagents for *Influenza A virus* cDNA amplification and subtypes A H5, H7, H9 differentiation using real-time hybridization and fluorescence detection. The fluorescent signal was detected using the Rotor-Gene Q amplifier (Qiagen, Germany).

RESULTS AND DISCUSSION

In 2019, the FGBI "ARRIAH" branch of the Republic of Crimea together with the Rosselkhoznadzor professionals arranged 5 expeditions, during which 8 Crimean raions were surveyed (Fig. 1) and 161 samples of pathological material from wild birds were collected (Table 1). The geographical sampling distribution is shown in Figure 1.

Wild waterfowl mass deaths were registered at Lake Donuzlav in February 2019. At the time significant congregations of Eurasian coots (*Fulica atra*) (about 8–10 thousand) were observed in this reservoir area. There were also gulls (*Larus*), ducks (*Anatinae*) and single mute swans (*Cygnus olor*) identified. A large number of Eurasian coot (*Fulica atra*) remains (about 1.5–2.0 thousand) (Fig. 2*a*, *b*) and, occasionally, remains of mallards (*Anas platyrhynchos*), common shelducks (*Tadorna tadorna*), black-necked grebes (*Podiceps nigricollis*), Caspian gulls (*Larus cachinnans*) (Fig. 2*c*) were found within the shoreline area.

In addition, Eurasian coots with clinical signs characteristic of the AI acute form and demonstrating lack of movement coordination, neck and head twisting (Fig. 3) were found on the shore [19–21].

No pathological and anatomical findings were observed during necropsy of Eurasian coot carcasses.

Nine wild bird carcasses were found in the Chernomorsky Raion at Lake Panskoye located 20 km from Lake Donuzlav (Table 1): Eurasian coot (*Fulica atra*) – 2, red-crested pochard (*Netta*) – 1, mallard duck (*Anas platyrhynchos*) – 4, Eurasian wigeon (*Mareca penelope*) – 1, greater scaup (*Aythya marila*) – 1. No wild bird mortality was recorded in other Crimean Raions. A decrease in the number of wild migratory birds was also noted during the field expeditions as compared to the previous years, especially on the Sivash Lagoon shore.

Fig. 2. Remains of Eurasian coots (Fulica atra) (a, b) and a Caspian gull carcass (c) at the Donuzlav Lake

Fig. 3. Eurasian coots demonstrating lack of movement coordination, head and neck twisting

A total of 13 samples of pathological material and 2 samples of wild bird faeces were taken during the expedition in February 2019 (Table 1). The AIV genome was not detected in these samples.

In August 2019 two field expeditions were arranged to the sites of semiaquatic bird congregation, during which the reservoirs of the Dzhankoysky, Chernomorsky and Saksky Raions of the Crimea were surveyed (Table 1). Testing of 77 faecal samples from wild birds and 3 pathological material samples (bird remains) showed absence of the AIV genome. During the survey of the above-mentioned areas it was noted that freshwater reservoirs became shallow and some of them dried up completely, degradation of aquatic vegetation was observed everywhere.

The freshwater lakes of the Chernomorsky and Saksky Raions, including the coast of the Karkinitsky Gulf of the Black Sea, were surveyed during the expedition conducted in October 2019. Two pathological material samples from a mute swan and 37 faecal samples from wild semiaquatic birds were collected (Table 1). The AIV genome was not detected in these samples.

The freshwater lakes in the Feodosiya Urban Okrug, Leninsky and Sovetsky Raions, as well as in the Sivash Lagoon area were surveyed on November 25, 2019. Wild semiaquatic birds were observed only at Lake Kuchuk-Ajigol in Feodosia Urban Okrug (Fig. 1). The real-time RT-PCR testing of 27 faecal samples collected in this territory showed that the influenza A subtype H9 virus genome was detected only in one of them.

In 2019 the Rosselkhoznadzor professionals also submitted 291 samples from birds shot in hunting areas and 548 samples from poultry from different Crimean Raions to the Laboratory and Diagnostic Center of the FGBI "ARRIAH" branch (the Republic of Crimea). The AIV genome was not detected in these samples.

In total 1,000 samples of pathological material were tested in the Laboratory for Molecular Diagnostics in 2019, among them 16.1% were samples obtained from wild semiaquatic birds, 29.1% – from wild birds shot in hunting areas and 54.8% – from poultry.

In 2020, the FGBI "ARRIAH" Branch in the Republic of Crimea, together with the Rosselkhoznadzor professionals organized 3 field expeditions (in winter-spring and autumn-winter periods) to wild waterfowl congregation sites, during which the following areas of the Crimean peninsula were surveyed: the shore of the Sivash Lagoon, freshwater lakes of the Feodosiya Urban Okrug, as well as Leninsky, Sovetsky, Nizhnegorsky, Dzhankoy raions (Fig. 4).

Fifty four samples from wild birds were collected for real-time RT-PCR testing, including 2 pathological material samples (the remains of a mute swan – Fig. 5*a* and a saker falcon – Fig. 5*b*) and 52 faecal samples from wild birds.

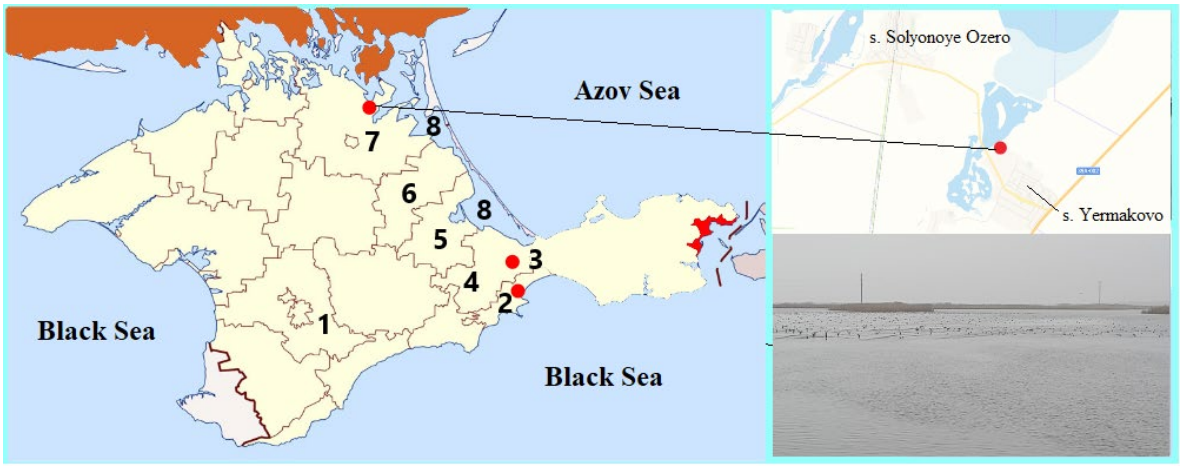

Fig. 4. Pathological and biological material sampling from semiaquatic wild birds in 2020

● *– location of sites for pathological material sampling from wild birds*

1. Simferopol
2. Feodosia Urban Okrug
3. Leninsky Raion
4. Kirovsky Raion
5. Sovetsky Raion
6. Nizhnegorsky Raion
7. Dzhankoysky Raion
8. Sivash Lagoon

The samples were taken on the shore of Lake Kuchuk-Ajigol (Feodosia Urban Okrug), at the Frontovoye reservoir (Leninsky Raion) and the freshwater lake shore near the Yermakovo settlement (Dzhankoysky Raion) (Table 2). No bird congregations were found in the other surveyed areas. Compared to 2019, the number of wild semiaquatic birds decreased, most likely, due to the decomposition of reedbeds, a decrease in the food supply for wild birds, changes in wintering and stopover sites during migration and nesting. The same trend was observed in previous years [4].

Table 2 presents data on the sampling site of pathological and biological material from wild semiaquatic birds, the number of samples taken, as well as the types and numbers of birds observed during field expeditions.

The genome of influenza A virus subtype H5 was detected in the pathological material collected from the remains of a mute swan in the shoreline area of a freshwater lake near the Yermakovo settlement, Dzhankoysky Raion, in December 2020 (Fig. 5*a*).

Fig. 5. Remains of a mute swan (a) and a saker falcon (b)

Table 2
Number and species of semiaquatic wild birds, number of samples in 2020

Sampling date	Bird species observed in the area	Number of birds	Number of collected samples
Frontline reservoir, Leninsky Raion			
February 04, 2020	Grey goose (*Anser anser*)	24	27
	White-fronted goose (*Anser albifrons*)	20	
	Black-necked grebes (*Podiceps nigricollis*)	200	
	Large cormorant (*Phalacrocorax carbo*)	70	
	Saker Falcon (*Falco cherrug*)	1	
Lake Kuchuk-Ajigol, Feodosia Urban Okrug			
November 03, 2020	Mute swan (*Cygnus olor*)	60	21
	Eurasian coot (*Fulica atra*)	400	
	Great bittern (Botaurus *stellaris*)	3	
	Grey heron (*Ardea cinerea*), Great egret (*Ardea alba*)	25	
	Red-headed pochard duck (*Aythya ferina*)	200	
	Greater scaup (*Aythya marila*)	15	
	Tufted duck (*Aythya fuligula*)	20	
	Northern shoveler (*Spatula clypeata*)	90	
	Eurasian teal (*Anas crecca*)	120	
	Gadwall (*Mareca strepera*)	38	
	Mallard duck (*Anas platyrhynchos*)	70	
The shore of a freshwater lake near the Yermakovo settlement, Dzhankoysky Raion			
December 18, 2020	Mute swan (*Cygnus olor*)	18	6
	Mallard duck (*Anas platyrhynchos*)	25	
	Eurasian coot (*Fulica atra*)	300	
		Total	54

Table 3
Number and location of biological material samples taken from poultry, pathological material samples taken from wild birds and animal product samples in 2020

Name of the material	Number of samples	Sampling site
Biological material (chicken droppings)	50	Yalta Urban Okrug, Belogorsky Raion
Biological material (chicken droppings)	50	Pervomaisky Raion
Biological material (chicken droppings)	200	Nizhnegorsky Raion
Biological material (chicken droppings)	25	Simferopol Raion
Biological material (chicken droppings)	100	Razdolnensky Raion
Biological material (chicken droppings)	150	Sudak city, Feodosia city, Feodosia Urban Okrug
Biological material (chicken droppings)	100	Kirovsky Raion
Biological material (chicken droppings)	100	Alushta Urban Okrug
Poultry biological material (cloacal swabs)	50	Yevpatoria Urban Okrug
Biological material (chicken droppings)	75	Sevastopol districts
Pathological material (wild bird carcasses)	14	Hunting areas in Sevastopol city
Pathological material (wild bird carcasses)	9	Hunting areas in Sevastopol city and Leninsky Raion
Pathological material (great bustard *Otis tarda* carcasses)	5	Hunting areas in Leninsky Raion
Frozen poultry meat (turkey) and frozen poultry offal (turkey)	12	Simferopol
Chicken eggs (30 pcs.)	6 pooled samples	Sevastopol city
Biological material (semiaquatic waterfowl faeces)	36	Lake Kuchuk-Ajigol, Feodosia Urban Okrug
Wild bird remains (Saker falcon)	1	
Biological material (semiaquatic waterfowl faeces)	11	Frontline reservoir, Leninsky Raion
Wild bird remains (a mute swan)	1	The shoreline of a freshwater lake near Yermakovo settlement, Dzhankoysky Raion
Biological material (semiaquatic waterfowl faeces)	5	
Total	1,000	

The genetic analysis was performed and the neuraminidase subtype (N8) of the identified influenza virus isolate was identified on the basis of the FGBI "ARRIAH" (Vladimir). The comparative genetic analysis of the AIV nucleic sequences of the 258 bp H gene fragment showed that the identified isolate belongs to the Asian genetic lineage of the highly pathogenic AIV subtype H5 (clade 2.3.4.4), which epidemically distributed in the countries of Asia, Europe, the Middle East and Africa in 2016–2020. According to the GenBank international sequence database, the A/duck/Egypt/SMG4/2019 (H5N8) virus isolated in Egypt in 2019 is the most genetically similar to the identified isolate (99.2–99.6% similarity). In addition, this isolate is genetically similar to H5N8 subtype AIVs detected in Omsk, Kurgan and Chelyabinsk Oblasts in 2020 (98.8–100.0% similarity), and H5N8 subtype AIVs isolated in 2016–2019 in the Voronezh, Rostov, Moscow Oblasts and the Republic of Kalmykia (97% similarity). The hemagglutinin cleavage site in the detected virus is -REKRRKR- which is characteristic of highly virulent AI pathogens. No AIV genome was detected in all the other tested samples.

Additionally, 28 samples collected from birds shot in hunting areas of the Leninsky Raion and Sevastopol city raions, 900 samples of biomaterial taken from poultry in the Belogorsky, Pervomaisky, Nizhnegorsky, Simferopolsky, Razdolnensky, Kirovsky Raions, the cities of Sudak, Yalta, Feodosia and the Urban Okrugs of Yalta, Feodosia, Sevastopol, as well as 18 samples of animal products marketed in Simferopol and Sevastopol were submitted to the Laboratory and Diagnostic Centre of the FGBI "ARRIAH" branch in the Republic of Crimea by the Rosselkhoznadzor professionals (Table 3). The AIV genome was not detected in these samples.

In total, 1,000 samples of pathological and biological material were tested for avian influenza using real time RT-PCR in 2020, out of them 8.2% were samples obtained from wild birds, 90% – from backyard poultry and 1.8% were samples of animal products.

CONCLUSION

Within the AI epidemiological monitoring in the territory of the Republic of Crimea in 2019–2020 nine areas were surveyed and 2,000 samples of pathological and biological material were tested, including 215 samples (10.75%) from wild semiaquatic birds, 319 samples (15.95%) from birds hunted in the hunting areas,

1,448 samples (72.4%) from poultry, 18 samples (0.9%) of products of animal origin.

The AIV genome was detected in two samples (0.1%) collected from wild birds: on the shore of Lake Kuchuk-Ajigol in Feodosia Urban Okrug (avian influenza A subtype H9) in November 2019 and on the lakeshore near the Yermakovo settlement, Dzhankoy Raion (avian influenza A subtype H5) in 2020. This fact is another confirmation that wild migratory birds present the main threat of AI introduction into the Crimean territory.

Therefore, a timely analysis of monitoring study results will allow monitoring the AI epidemic situation and, in case of a threat, taking timely measures to prevent the AIV spread, contain outbreaks and carry out measures aimed primarily at breaking the transmission chain.

The prospect of further research is to conduct consistent and detailed AI epidemiological monitoring on the Crimean peninsula with the coverage expansion of the studied areas (hunting areas, preserve areas).

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