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SEROLOGICAL MONITORING OF AVIAN INFLUENZA VIRUSES SUBTYPES H5 AND H7 IN WILD BIRDS IN THE AZOV-BLACK SEA REGION

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To date, influenza remains an unpredictable infection for animals, birds and people. In Ukraine in National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine» since 2000 it was conducted monitoring study on wild bird circulation of influenza viruses in their populations. Special attention was paid to the circulation of H5 and H7 subtypes of avian influenza viruses, which can be potentially dangerous for poultry. By the results of serologic studies antibodies to avian influenza virus of H5 subtype were detected in the blood serum only in 2003 and 4.93 % As for egg yolks, then also in 2003, egg yolks of 16.6 % had antibodies to avian influenza virus of H5 subtype in small diagnostic titers. It was established that in 2006–2011 antibodies to avian influenza virus of H5 subtype were detected in blood serum of Mallards in 2007, 2008, 2011 in Kherson Region (seroprevalence of 1.55–4.00 %) and Zaporozhye Region (seroprevalence 11.29 %). Antibodies to influenza virus of H7 subtype were detected only in 2011 at 2.32 % wild duck. It should be noted that the antibody levels were not high 1:16–1:32 (4–5 $\log_2$).

Keywords: avian influenza viruses, wild birds, antibody, subtype H5, H7.

To date, influenza virus remains unpredictable infection for animals, birds and people. The constant emergence of new strains and variants with new properties and pathogenicity to new host requires constant monitoring and careful research of new viruses. Taking into account that the main and primary reservoir of influenza viruses in nature is wild birds, especially waterfowl and shorebirds undoubtedly is to conduct continuous epizootic monitoring in populations of these birds.

In general, it is reported that avian influenza viruses isolated from wild birds, belonging to over 100 species of 12 orders. There were isolated influenza viruses with all known subtypes of hemagglutinin (H1–H16) and neuraminidase subtypes (N1–N9) (6) from wild birds. Most of the species from which it was isolated influenza viruses belonging to the *Anseriformes* and *Charadriiformes*. Of the variety of *Anseriformes* most influenza viruses isolated from ducks from the *Anatinae*.

As for *Charadriiformes*, the highest number of avian influenza viruses has been isolated from representatives of *Scolopacidae* families (3), *Laridae* and a small amount of *Alcidae*. In addition to representatives of the above-mentioned orders from representatives of other water birds and shorebirds: *Ciconiiformes* (4), *Gaviiformes, Gruiformes, Pelecaniformes, Podicipediformes, Procellariiformes* it has been isolated avian influenza viruses, but the amount received positive samples from them is generally not large. Besides, there are several reports on allocation of influenza viruses from wild birds orders *Columbiformes, Piciformes* and *Passeriformes, Galliformes*, which are usually related to terrestrial birds, but the number of such messages is very small.

Thus, to date, is fully proven that wild birds are the natural reservoir of low-path avian influenza viruses and it is a considerable role in their distribution. However, it remains unresolved questions of role of wild birds in the spread of highly pathogenic influenza viruses, especially over long distances in other geographic regions (2).

Most of the highly-pathogenic variants of virus refers to influenza A viruses of H5 and H7, so with point of view of danger to the poultry these viruses deserve special attention and require careful monitoring circulation. But recently, influenza viruses of these subtypes acquired great importance also for human health. Over the past 10 years worldwide were reported several outbreaks and human cases of avian influenza virus. It should be noted that these cases were accompanied by lots of people (over 60 %) deaths. First of all these are dangerous to human health viruses include avian influenza viruses H5N1. Thus, according to the World Health Organization at the beginning of 2014 the whole world 650 people were infected with highly pathogenic avian influenza virus N5N1, of whom 386 were dead in 15 countries (1).

In spring 2013 a new threat to human health were a new avian influenza viruses H7N9. The first and only cases of human influenza virus of this subtype that has been recorded in China. It should be noted that by this time there were no reported cases of human influenza virus of this subtype. When people infection, these viruses cause severe infection, which can lead to death. To date, two waves of people infection were recorded; in general there were infected 207 people, some of whom were dead. This virus is low pathogenic for poultry, and this infection usually occurs without overt clinical signs. Besides, it is remains unclear the reservoir and source of the virus.

All above listed demonstrates the relevance of monitoring of avian influenza subtypes H5 and H7 circulating in the Azov-Black Sea region. This region is a unique place of intersection of migration routes of many species of wild birds. This migration routes it relates to Europe, Asia, Africa, so the risk of introduction of new genetic variants of influenza virus of particularly emergent H5 and H7 subtypes, is large. Therefore, the main objective of our research was to monitor the circulation of influenza viruses of H5 and H7 subtypes in wild bird populations of different ecological groups of Azov-Black Sea region involving serological, virological and molecular genetic methods and determine origin and phylogenetic relationships of these viruses.

Materials and methods. ***Wild bird surveillance.*** Wild bird surveillance were conducted from 2001 to 2011 in the Azov-Black Sea region of Ukraine, which is one of strenuous in ornithological regard to the location of Eastern Europe, a place of passage, stops during migration, breeding of migratory and other bird species with very high biodiversity. There are intersecting areas of flight of birds from the Baltic and the Caspian to the Black and Mediterranean seas, from Western Siberia and Kazakhstan to Western Europe and North Africa. In some years, the number of wild birds during migration and nesting exceeded 1 million individuals.

Since 2001 to 2011 it was conducted serological monitoring of the presence of antibodies to influenza viruses of different subtypes, including H5 and H7. Since 2006 to 2011 for the study there were obtained 708 serum samples and 238 samples of egg yolks of wild birds of 44 species of 7 order (*Pelecaniformes, Ciconiiformes, Anseriformes, Gruiformes, Charadriiformes, Passeriformes, Columbiformes*).

Serological studies. Detection of antibodies to avian influenza virus subtypes H5 and H7 were performed in serum and egg yolks of wild birds by HI test and ELISA. Sampling of blood from wild birds carried on trapped birds by methods described in the recommendations of the OIE. Blood serum of wild birds was received by conventional methods. The eggs of wild birds we collected in nesting sites in the amount of 1–2 eggs from the nest, depending on the size of the stack. For research we use not hatched eggs. Preparation of egg yolks of wild birds carried out by the following procedure, adapted for the study of wild birds and patented in Ukraine : yolk was separated from the protein and thoroughly mixed with PBS (pH 7,2–7,4) in a ratio of 1:1, to the mixture was added an equal volume of chloroform. The mixture was carefully for 5–10 minutes, and subjected to centrifugation at 3000 g 15 minutes. After centrifugation, the supernatant was used for research just as serum. HI test maintenance with blood serum and egg yolks of wild birds in monitoring studies were performed using inactivated antigens of influenza virus subtypes H5N1 and H7N1 (produced by NSC «IECVM»). For the serological tests of blood sera from experimentally infected chickens were used inactivated homologous virus A/teal/Djankoy/4-17-11/2010 H5N2, A/mallard/Askania Nova/23-15-02/2011 H7N3 as antigens. The hemagglutination (HA) assay and HI test were performed in V-bottom 69-well microtiter plates using standard protocol recommended by the OIE (5). Detection of antibodies to avian influenza virus in by ELISA was performed using commercial test kits: ELISA test kits: ID Screen ® Influenza A Antibody Competition (produced by ID VET, France), FlockChek ® (MultiS-Screen) Avian influenza virus antibody test kit ELISA, IDEXX, USA.

Results. In Ukraine in National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine» since 2000 it was conducted monitoring study on wild bird circulation of influenza viruses in their populations. Special attention was paid to the circulation of H5 and H7 subtypes of avian influenza viruses, which can be potentially dangerous for poultry.

The study blood serum and egg yolks of wild birds of Azov-Black Sea region on the presence of antibodies to avian influenza virus subtypes H5 and H7 were conducted since 2001 to 2011. Since 2001 to 2004 there were examined 228 serum samples and 87 samples of egg yolks from wild birds of 4 species (Mallard, Ruddy Shelduck, Greylag Goose, White-fronted Goose) order Anseriformes. By the results of serologic studies antibodies to avian influenza virus of H5 subtype were detected in the blood serum only in 2003 and 4.93 % Mallards and Ruddy Shelduck. In wild waterfowl and other species in other years the antibodies to avian influenza virus of H5 and H7 subtype have not been identified. As for egg yolks, then also in 2003, egg yolks of 16.6 % Mallards and Ruddy Shelduck had antibodies to avian influenza virus of H5 subtype in small diagnostic titers. In other years, antibodies have not been identified.

A larger scale study the antibodies to influenza A viruses of different subtypes (H1–H14) and different serotypes of avian paramixoviruses (APMV 1-9) in serum and egg yolk of wild birds were held in 2006–2011 in Kherson, Zaporizhzhya, Sumy, Odessa, Kharkiv regions and Crimea. The results of studies blood serum and egg yolks on the presence of antibodies to avian influenza virus of subtypes H5 and H7 are shown in Table 1.

Table 1 – The results of serological tests of serum from wild birds concerning the presence of antibodies to avian influenza virus of H5 and H7 subtypes

Year	Bird species	Samples, total	Influenza virus subtypes	Number of serum samples having antibodies to influenza virus in HIT in appropriate dilution, log_2						
				Negative	≤3	4	5	6	7	≥8
Kherson region										
2007	Mallard	50	H5	48	-	2	-	-	-	-
2008	Mallard	86	H5	84	-	2	-	-	-	-
2011	Mallard	129	H5	127	-	2	-	-	-	-
			H7	126	-	3	-	-	-	-
AR Crimea										
2007	Common Tern	7	H5	4	-	3	1	-	-	-
Odessa region										
2008	Mute Swan	13	H5	11	-	1	1	-	-	-
Zaporizhzhya region										
2009	Avocet	1	H5	0	-	-	1	-	-	-
	Mallard	62	H5	53	2	6	1	-	-	-

* Titers less than or equal to 3 log2 were considered as not specific

It was established that in this period antibodies to avian influenza virus of H5 subtype were detected in blood serum of Mallards in 2007, 2008, 2011 in Kherson Region (seroprevalence of 1.55–4.00 %) and Zaporozhye Region (seroprevalence 11.29 %). Antibodies to influenza virus of H7 subtype were detected only in 2011 at 2.32 % Mallards. It should be noted that the antibody levels were not high 1:16–1:32 (4–5 $\log_2$).

The different results were obtained at study egg yolks of wild birds (Table 2).

Table 2 – The results of HI tests of egg yolks from wild birds on the presence of antibodies to avian influenza virus of H5 and H7 subtypes

Year	Bird species	Samples, total	Influenza virus subtypes	Number of serum samples having antibodies to influenza virus in HIT in appropriate dilution, $\log_2$						
				Negative	≤3*	4	5	6	7	≥8
Kherson region (Askania-Nova)										
2006	Mallard	24	H5	15	7	-	2	-	-	-
	Coot	1	H5	0	1	-	-	-	-	-
	Ruddy Shelduck	7	H5	5	2	-	-	-	-	-
	Yellow-legged Gull	13	H5	11	-	2	-	-	-	-
			H7	11	2	-	-	-	-	-
2009	Mallard	24	H5	23	-	-	1	-	-	-
	Yellow-legged Gull	22	H5	17	-	3	-	-	-	2
			H7	19	-	2	1	-	-	-
2010	Yellow-legged Gull	15	H5	13	-	1	1	-	-	-
AR Crimea (Sivash bay)										
2006	Cormorant	10	H5	5	4	1	-	-	-	-
	Mediterranean Gull	9	H7	6	3	-	-	-	-	-
2007	Coot	3	H5	2	-	-	-	-	-	1
2008	Mediterranean Gull	4	H7	3	-	1	-	-	-	-
	Common Tern	1	H5	0	-	-	-	-	-	1
			H7	0	-	-	1	-	-	-
Kharkiv region										
2007	Black-headed Gull	2	H5	0	-	2	-	-	-	-
	Bluethroat	1	H5	0	-	1	-	-	-	-
2009	Mallard	4	H5	2	-	-	-	1	-	1

* Titers less than or equal to 3 log were considered as not specific

Antibodies to influenza virus of H5 subtype were found in Mallards, Coots, Yellow-legged Gulls, Cormorants in 2006, 2007, 2009, 2010 in Kherson, Kharkiv regions and AR Crimea, prevalence was averaged 18.33 % (4.16 – 22 72 %). The majority of samples had antibody level 1:16–1:32 (4–5 $\log_2$), but some samples had high antibody levels, there were recorded 1:256 or more (≥ 8 $\log_2$). When analyzing the research results it was revealed that the number of samples having antibodies to influenza virus of H7 subtype was significantly less. So, antibodies to the virus of this subtype were detected only in Yellow-legged Gulls in 2006, 2009, in Mediterranean Gulls and Black-headed Gulls in 2008 in the Crimea and Kherson region. Seroprevalence was ranged from 13.68 to 25.0 %.

Conclusions. The results indicate circulation of low pathogenic avian influenza viruses subtype H5 and H7 in wild waterfowl populations in the Azov-Black Sea region. These findings support the need for ongoing monitoring of avian influenza for early prevention of highly pathogenic variants of viruses that may pose a threat to poultry.

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СЕРОЛОГІЧНИЙ МОНІТОРИНГ ГРИПУ ПТИЦІ ПІДТИПІВ H5 ТА H7 СЕРЕД ДИКИХ ПТАХІВ АЗОВО-ЧОРНОМОРСЬКОГО РЕГІОНУ

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На сьогоднішній день, грип залишається непередбачуваною інфекцією для тварин, птахів і людей. В Україні в Національному науковому центрі «Інститут експериментальної і клінічної ветеринарної медицини» з 2000 року було проведено моніторингове дослідження щодо циркуляції вірусів грипу серед диких птахів. Особливу увагу було приділено циркуляції підтипів H5 і H7 вірусів грипу птиці, які можуть бути потенційно небезпечні для свійських птахів. За результатами серологічних досліджень антитіл до вірусу грипу птиці підтипу H5 були виявлені в сироватці крові тільки у 2003 році (кількість позитивних проб 4,93 %). Що стосується наявності антитіл жовтках яєць диких птахів, то 16,6 % проб у 2003 році мали антитіла до вірусу грипу підтипу H5 у невеликих діагностичних титрах. Пізніше антитіла до вірусу грипу птиці підтипу H5 були виявлені в сироватці крові диких качок у 2007, 2008, 2011 роках у Херсонській області (кількість позитивних проб 1,55–4,00 %) і Запорізькій області (кількість позитивних проб 11,29 %). Антитіла до вірусу грипу H7 підтипу були виявлені тільки у 2011 році у 2,32 % диких качок. Слід зазначити, що рівні антитіл були невисокі 1: 16–1: 32 (4–5 log_2).

Ключові слова: грип птиці, дикі птиці, підтипу H5, H7.

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ЕКОЛОГО-ЕПІДЕМІОЛОГІЧНІ ТА СОЦІАЛЬНО-ЕКОНОМІЧНІ АСПЕКТИ ЗООНОЗНИХ ПРИРОДНО-ОСЕРЕДКОВИХ ІНФЕКЦІЙ НА ПІВДНІ УКРАЇНИ

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Представлені дані щодо широкої розповсюдженості зоонозів (орнітозу, туляремії, лептоспірозу, арбовірусних інфекцій) серед птахів, тварин і людей на півдні України. Встановлена наявність природних, антропургічних, поліінфектних осередків особливо небезпечних інфекцій різної етіології. Розроблено комплекс науково обґрунтованих рекомендацій з удосконалення системи профілактики зоонозних природно-осередкових інфекцій.

Ключові слова: зоонози, природні осередки, люди, птахи, ссавці, діагностика, профілактика.

Зоонозні природно-осередкові особливо небезпечні інфекції (ОНІ) є серйозною проблемою національних служб охорони здоров'я та ветеринарної медицини внаслідок їх глобального розповсюдження, негативного впливу на здоров'я населення, економіку народного господарства при відсутності діючої системи профілактики [1–6].

Моніторинг ряду природно-осередкових ОНІ необхідний в зв'язку з тим, що їх збудники розглядаються в якості потенційних агентів біологічної зброї і дані про них вкрай важливі для створення ефективної системи протиепідемічного захисту. Вищезазначене відноситься до різних ОНІ, в тому числі орнітозу та туляремії, збудниками яких є *Chlamydophila psittaci* (*C. psittaci*) і *Francisella tularensis* (*F. tularensis*), відповідно [7, 8].

Туляремія – найважливіший зооноз [9], що має природно-осередковий характер з широким кругом джерел, численних переносників, різноманітністю шляхів передачі інфекції. Орнітоз належить до групи природно-антропургічних зоонозів з переважно респіраційним шляхом передачі інфекції, різною течією інфекційного процесу – від гострої смертельної форми до латентного носійства [3–5, 10].

До збудників туляремії та орнітозу сприйнятливість людини дуже висока, практично 100 %, завдяки чому *F. tularensis* віднесена до найбільш небезпечних патогенних мікроорганізмів (вища категорія «А»), а *C. psittaci* – до категорії «В» [7, 8]. Екологічна пластичність, здатність до персистенції збудників туляремії та орнітозу, полігостальність, стійкість природних осередків зумовлює періодичні епідускладнення різного масштабу – від спорадичних випадків до групових захворювань [3–5, 11, 12, 13].

В Україні епідеміологічна значимість зоонозних природно-осередкових ОНІ в різні роки була неоднаковою і залежала від соціально-економічних умов, рівня діагностики, масштабу проведених протиепізоотичних, протиепідемічних заходів, координації дій фахівців медичної та ветеринарної служб.