AVIAN DISEASES 56:1010–1016, 2012

Research Note—

Avian Influenza Virus Wild Bird Surveillance in the Azov and Black Sea Regions of Ukraine (2010–2011)

Denys Muzyka,[AC] Mary Pantin-Jackwood,[B] Erica Spackman,[B] Borys Stegniy,[A] Oleksandr Rula,[A] and Pavlo Shutchenko[A]

[A]National Scientific Center ''Institute of Experimental and Clinical Veterinary Medicine'', Kharkiv, 61023, Ukraine
[B]Southeast Poultry Research Laboratory, USDA/ARS, Athens, GA 30605

Received 11 April 2012; Accepted 16 July 2012; Published ahead of print 17 July 2012

SUMMARY. The Azov and Black Sea basins are part of the transcontinental wild bird migration routes from Northern Asia and Europe to the Mediterranean, Africa, and Southwest Asia. These regions constitute an area of transit, stops during migration, and nesting for many different bird species. From September 2010 to September 2011, a wild bird surveillance study was conducted in these regions to identify avian influenza viruses. Biological samples consisting of cloacal and tracheal swabs and fecal samples were collected from wild birds of different ecological groups, including waterfowl and sea- and land-based birds, in places of mass bird accumulations in Sivash Bay and the Utlyuksky and Molochniy estuaries. The sampling covered the following wild bird biological cycles: autumn migration, wintering, spring migration, nesting, and postnesting seasons. A total of 3634 samples were collected from 66 different species of birds. During the autumn migration, 19 hemagglutinating viruses were isolated, 14 of which were identified as low pathogenicity avian influenza (LPAI) virus subtypes H1N?, H3N8, H5N2, H7N?, H8N4, H10N7, and H11N8. From the wintering samples, 45 hemagglutinating viruses were isolated, 36 of which were identified as LPAI virus subtypes H1N1, H1N? H1N2, H4N?, H6N1, H7N3, H7N6, H7N7, H8N2, H9N2, H10N7, H10N4, H11N2, H12N2, and H15N7. Only three viruses were isolated during the spring migration, nesting, and postnesting seasons (serotypes H6, H13, and H16). The HA and NA genes were sequenced from the isolated H5 and N1 viruses, and the phylogenetic analysis revealed possible ecological connections between the Azov and Black Sea regions and Europe. The LPAI viruses were isolated mostly from mallard ducks, but also from shellducks, shovelers, teals, and white-fronted geese. The rest of the 14 hemagglutinating viruses isolated were identified as different serotypes of avian paramyxoviruses (APMV-1, APMV-4, APMV-6, and APMV-7). This information furthers our understanding of the ecology of avian influenza viruses in wild bird species.

RESUMEN. *Nota de Investigación*—Vigilancia para el virus de la influenza aviar en aves silvestres en las regiones del Mar de Azov y del Mar Negro en Ucrania (años 2010–2011).

Las cuencas del Mar de Azov y del Mar Negro son parte de las rutas transcontinentales de aves silvestres migratorias procedentes del norte de Asia y Europa hacia el Mediterráneo, África y el suroeste de Asia. Estas regiones constituyen un área de tránsito, de descanso durante la migración y de anidación para varias especies de aves diferentes. Desde septiembre 2010 hasta septiembre 2011, un estudio de vigilancia de aves silvestres se llevó a cabo en estas regiones con el fin de identificar los virus de la influenza aviar. Las muestras biológicas que consistieron de hisopos cloacales y traqueales y de muestras fecales de aves silvestres de diferentes grupos ecológicos, incluyendo aves acuáticas además de aves marinas y terrestres, en los lugares de acumulación masiva en la Bahía de Sivash y en los estuarios de Utlyuksky y Molochniy. El muestreo abarcó los siguientes ciclos biológicos de aves silvestres: la migración de otoño, el descanso invernal, la migración de primavera, temporada de anidación, y las estaciones posteriores a la anidación. Se recolectaron un total de 3634 muestras de 66 especies diferentes de aves. Durante la migración otoñal, se aislaron 19 virus hemaglutinantes de los cuales 14 fueron identificados como virus de influenza aviar de baja patogenicidad, de los subtipos H1N?, H3N8, H5N2, H7N?, H8N4, H10N7 y H11N8. De las muestras de invierno, se aislaron 45 virus hemaglutinantes, 36 de los cuales fueron identificados como virus de baja patogenicidad subtipos H1N1, H1N? H1N2, H4N?, H6N1, H7N3, H7N6, H7N7, H8N2, H9N2, H10N7, H10N4, H11N2, H12N2 y H15N7. Sólo tres virus fueron aislados durante la migración de primavera, de anidación, y de las estaciones posteriores a la anidación (serotipos H6, H13, y H16). Los genes HA y NA se secuenciaron de los virus aislados con subtipos H5 y N1, y el análisis filogenético reveló posibles conexiones ecológicas entre los mares de Azov y las regiones del Mar Negro y Europa. Los virus de baja patogenicidad fueron aislados en su mayoría de patos de collar, pero también en patos tarros de blancos, patos pico de cuchara, cercetas y gansos de frente blanca. El resto de los 14 virus hemaglutinantes aislados fueron identificados como diferentes serotipos de paramixovirus aviar (APMV-1, APMV-4, APMV-6, y APMV-7). Esta información amplía el conocimiento de la ecología de los virus de influenza aviar en las especies de aves silvestres.

Key words: avian influenza, avian influenza virus, surveillance, wild birds, Crimea

Abbreviations: AI = avian influenza; AIV = avian influenza virus; APMV = avian paramyxovirus; HA = hemagglutinating; HP = highly pathogenic; LP = low pathogenicity; NA = neuraminidase; nt = nucleotide

Avian influenza viruses (AIVs) are type A influenza viruses belonging to the family *Orthomyxoviridae* (13). AIVs are divided into subtypes based on the two surface glycoproteins, the HA (subtypes H1–H16) and the NA (subtypes N1–N9). Because of their segmented genome, AIVs can reassort and consequently lead to the emergence of new virus strains. Wild aquatic birds are the natural reservoirs of AIVs and play an important role in the epizootology of avian influenza (AI) (13). Low pathogenicity (LP) AIVs are maintained in wild bird reservoirs, predominately the aquatic birds in the orders Anseriformes and Charadriiformes (9,15). These viruses are passed within and between species of birds that share the same ecosystems, and such infections are typically not associated with disease (14). In rare cases isolates of the Asian H5N1 highly

[C]Corresponding author. E-mail: dmuzyka77@gmail.com

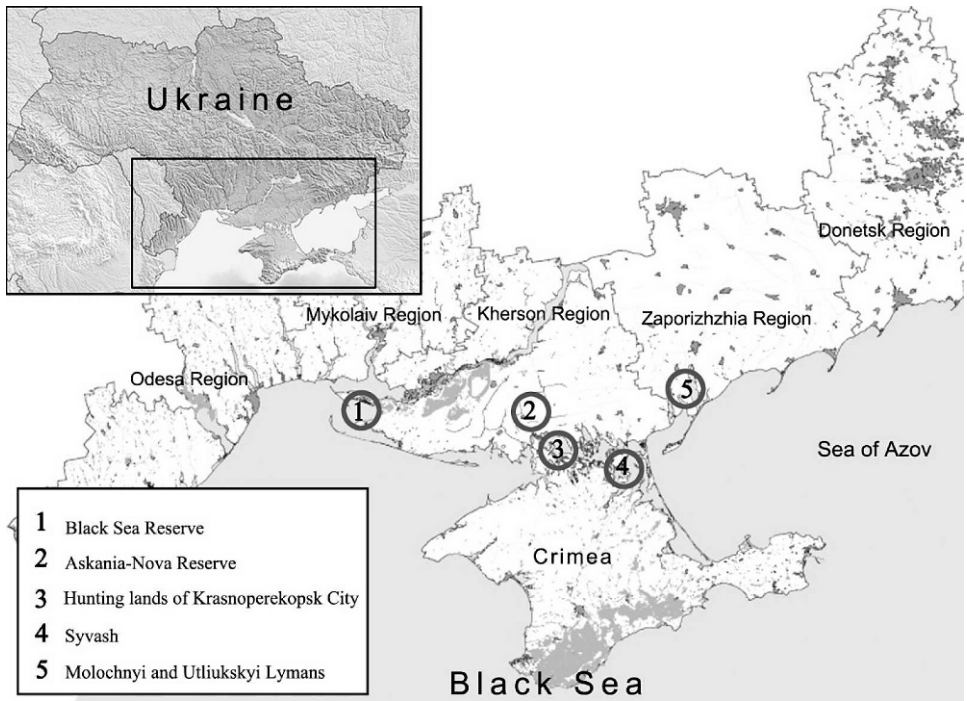

Fig. 1. Map of Ukraine indicating the regions included in the AIV wild bird surveillance study.

pathogenic (HP) AIV lineage have caused disease and mortality in some wild bird species (2). Poultry species (chickens, turkeys, quail, guinea fowl, etc.) reared in proximity to wild bird ecosystems can become infected by viruses carried by the wild birds.

Commercial poultry is an important industry in Ukraine; in addition backyard poultry is common in the southern regions. It is very important for Ukraine to remain free of AI, and one of the components for AI control is to monitor AIV infections in wild bird populations. The importance of conducting AIV surveillance in wild birds became evident during the outbreaks of H5N1 HPAI in Ukraine in 2005, 2006. and 2008, when the virus was introduced into the country by wild birds during the autumn migration (16). The role of wild birds was considerable in the further dissemination of H5N1 HPAIV into territories of Autonomous Republic of Crimea. Due to a very cold winter in 2005–2006, and other unfavorable weather factors, wild birds constantly moved in many directions, dispersing the virus and originating new outbreaks in the different geographic areas of Crimea (7,11).

Ukraine occupies an unique geographical location in Central and Eastern Europe, as the transcontinental migrations of numerous wild bird species pass from North Asia and Europe to the Mediterranean, Africa, and Southwest Asia and cross from the Baltic and Caspian seas to the Black and Mediterranean seas, and from Western Siberia and Kazakhstan to Western Europe and North Africa.

The Azov and Black Sea regions were selected for monitoring wild birds for AIV and avian paramyxovirus (APMV) infections because of the abundance of migratory birds. The Azov and Black Sea basins and the peninsula of Crimea are historically areas of nesting, flight, migratory stops, and wintering for many bird species. The Azov–Black Sea coast is one of the densest territories of Eastern Europe from an ornithological point of view. In Ukraine there are 19 wetlands of international importance with an area of more than 590,000 ha. More than 320 types of birds inhabit the wetlands, representing almost 84% of bird species in Ukraine. In this study we present the results of wild bird AIV and APMV surveillance in the Azov and Black Sea regions of Ukraine between September 2010 and September 2011.

MATERIALS AND METHODS

Wild bird surveillance. Sampling of wild birds was conducted from September 2010 to September 2011 in the following regions of the Azov and Black Sea regions: Sivash Bay, Utliukskyi and Molochnyi estuaries, Askania-Nova Reserve, Black Sea Biosphere Reserve, and hunting lands of Krasnoperekopskyi, Nyznygorsky, and Djankoysky districts of Crimea (Fig. 1). The collection of biological material from wild birds covered the following annual bird life cycles: autumn migration (September to November 2010), wintering (December 2010 to February 2011), and spring migration, nesting, and postnesting movements (March 2011 to August 2011).

Sample collection. Sampling from wild birds was carried out in cooperation with ornithologists, who helped determine the bird species identity. Cloacal and tracheal swabs were collected from captured birds and from birds shot by hunters. Fresh feces were collected from certain species of birds in places of mass bird accumulations. Feces were collected only if the origin and type of bird had been established. Samples of feces were taken in a checkerboard pattern at a distance of at least 1.5–2 m from each other, to avoid selecting feces from the same bird.

The samples (cloacal swabs, tracheal swabs, and feces) were taken from adult birds, regardless of gender. The sample size depended on the size of the flock and was at least 25 samples if the flock was up to 500 birds and at least 50 samples per 1000 birds if the number of birds in the flock was more than 1000. Estimates of the number of birds were conducted by ornithologists.

Samples were collected in cryotubes containing 1.0 ml of transport media (phosphate-buffered saline:glycerin, 1:1) with antibiotics (penicillin 2000 U/ml, streptomycin 2 mg/ml, gentamicin 50 μg/ml, and nystatin 1000 U/ml), and a fivefold concentration of antibiotics was used for the fecal samples and cloacal swabs (5). Samples were stored at −196 C in liquid nitrogen, where they were kept until processing.

Virus isolation. Virus isolation was conducted in accordance with the OIE procedures (5). Cloacal swabs, tracheal swabs, and fecal samples were inoculated into the allantoic cavity of 9–10-day-old SPF chicken embryonated eggs. Every sample was passaged three times. The presence of hemagglutinating (HA) viruses in allantoic fluid was determined by the hemagglutination test with a 1% suspension of chicken red blood cells (5).

Virus identification. The HA virus subtype was determined by hemagglutination inhibition tests as previously described (1,5). Determi-

Table 1. Number of samples of biological material and the results of virus isolation data (total/number positive) of wild birds of different ecological groups in the central part of the Azov and Black Sea regions from September 2010 to September 2011.

Bird species	Sampling periods			Total samples (%)
	Autumn migration	Wintering	Spring migration, nesting, postnesting movements	
Pelecaniformes				
Cormorant (*Phalacrocorax carbo*)	0/15	—	—	0/15
Ciconiiformes				
Gray heron (*Ardea cinerea*)	—	0/4	0/1	0/5
Purple heron (*Ardea purpurea*)			0/1	0/1
Anseriformes				
Mute swan (*Cygnus olor*)	0/1	—	—	0/1
White-fronted goose (*Anser albifrons*)	0/216	9/649 H9N2(3), H6N1(6)[A]	—	9/865 (1.04)[B]
Graylag goose (*Anser anser*)	—	0/20	—	0/20
Branta ruficollis (*Rufibrenta ruficollis*)	—	0/215	—	0/215
Shelduck (*Tadorna tadorna*)	1/8 H8N4	7/238 H1N2, H1N? (4), H11N2 (2)	—	8/246 (3.25)
Ruddy shelduck (*Tadorna ferruginea*)	0/90	4/190 H4N?, H11N2 (2), H11N6	—	4/280 (1.42)
Wild duck[C]	0/4	2/45 H8N2, H10N4	—	2/49 (4.08)
Mallard duck (*Anas platyrhynchos*)	11/366 H1N?, H3N8 (5), mixH3/H4N8, H7N?, H8N4, H10N7, H11N8	13/392 H1N1(2), H7N3 (3), H7N6 (2), H7N7, H10N7 (2), mixH10/APMV-7, H12N2/N8, H15N7	—	24/758 (3.16)
Red-crested pochard (*Netta rufina*)	0/1	—	—	0/1
Wigeon (*Anas penelope*)	0/130	—	—	0/130
Pochard (*Aythya ferina*)	0/3	—	—	0/3
Pintail (*Anas acuta*)	0/3	—	—	0/3
Garganey (*Anas querquedula*)	0/3	—	—	0/3
Teal (*Anas crecca*)	1/18 H5N2	—	0/1	1/19 (5.26)
Shoveler (*Anas clypeata*)	1/4 H8N4	—	—	1/4 (25.0)
Gadwall (*Anas strepera*)	0/3	—	—	0/3
Galliformes				
Gray partridge (*Perdix perdix*)	0/3	0/1	—	0/4
Gruiformes				
Crane (*Grus grus*)	0/7	—	—	0/7
Coot (*Fulica atra*)	0/34	—	0/1	0/34
Water rail (*Rallus aquaticus*)	—	0/1		0/1
Moorhen (*Gallinula chloropus*)	—	—	0/2	0/2
Little crake (*Porzana parva*)	—	—	0/2	0/2
Charadriiformes				
Yellow-legged gull (*Larus cachinnans*)	0/9	1/39 H1/H5	1/42 (H6N?)	2/90 (2.22)
Slender-billed gull (*Larus genei*)	0/12	—	0/32	0/44
Mediterranean gull (*Larus melanocephalus*)	—	0/2	0/141	0/143
Common gull (*Larus canus*)	—	0/9		0/9
Black-headed gull (*Larus ridibundus*)	—	—	1/131 (H16N?)	1/131 (0.76)
Sandwich tern (*Thalasseus sandvicensis*)	0/2	—	0/15	0/17
Gull-billed tern (*Gelochelidon nilotica*)	—	—	0/85	0/85
Gray plover (*Pluvialis squatarola*)	0/4	—	0/5	0/9
Kentish plover (*Charadrius alexandrinus*)	0/1	—	—	0/1
Sanderling (*Calidris alba*)	0/3	—	—	0/3
Dunlin (*Calidris alpina*)	0/23	—	0/2	0/25
Little stint (*Calidris minuta*)	—	—	0/2	0/2
Temminck's stint (*Calidris temminckii*)	—	—	0/3	0/3

Table 1. Continued.

Bird species	Sampling periods: Autumn migration	Wintering	Spring migration, nesting, postnesting movements	Total samples (%)
Common sandpiper (*Actitis hypoleucos*)	—	—	0/5	0/5
Wood sandpiper (*Tringa glareola*)	—	—	1/37 (H13N?)	1/37 (2.70)
Green sandpiper (*Tringa ochropus*)	—	—	0/5	0/5
Marsh sandpiper (*Tringa stagnatilis*)	—	—	0/1	0/1
Greenshank (*Tringa nebularia*)	—	—	0/6	0/6
Black-winged stilt (*Himantopus himantopus*)	—	—	0/4	0/4
Oystercatcher (*Haematopus ostralegus*)	—	—	0/4	0/4
Collared pratincole (*Glareola pratincola*)	—	—	0/1	0/1
Snipe (*Gallinago gallinago*)	—	—	0/1	0/1
Ruff (*Phylomachus pugnax*)	—	—	0/91	0/91
Coraciiformes				
Kingfisher (*Alcedo atthis*)	—	—	0/3	0/3
Passeriformes				
Sand martin (*Riparia riparia*)	—	—	0/3	0/3
Swallow (*Hirundo rustica*)	—	—	0/14	0/14
Jackdaw (*Corvus monedula*)	—	0/10	—	0/10
Calandra lark (*Melanocorypha calandra*)	—	0/60	—	0/60
Pied wagtail (*Motacilla alba*)	—	—	0/1	0/1
Yellow wagtail (*Motacilla flava*)	—	—	0/2	0/2
Magpie (*Pica pica*)	—	0/35	—	0/35
Rook (*Corvus frugilegus*)	—	—	0/30	0/30
Chaffinch (*Fringilla coelebs*)	—	0/2	—	0/2
Reed bunting (*Emberiza schoeniclus*)	—	0/20	0/1	0/21
Starling (*Sturnus vulgaris*)	—	0/35	0/2	0/37
Reed warbler (*Acrocephalus scirpaceus*)	—	—	0/5	0/5
Great reed warbler (*Acrocephalus arundinaceus*)	—	—	0/11	0/11
Sedge warbler (*Acrocephalus schoenobaenus*)	—	—	0/1	0/1
Savi's warbler (*Locustella luscinioides*)	—	—	0/1	0/1
Bearded tit (*Panurus biarmicus*)	—	—	0/8	0/8
Icterine warbler (*Hippolais icterina*)	—	—	0/1	0/1
Olivaceous warbler (*Hippolais pallida*)	—	—	0/1	0/1
Total, no. (%)	14/962 (1.45)	36/1966 (1.83)	3/706 (0.42)	53/3634 (1.46)

[A]Total number/number of isolated viruses, HA:NA (number of viruses of this subtype).
[B]Percentage of positive samples.
[C]The species could not be identified because of bad weather.

nation of neuraminidase (NA) subtype was conducted by the neuraminidase activity inhibition test (8). For these studies, the following antisera were used: H1N1, H2N3, H3N8, H4N6, H5N1, H6N8, H7N1, H8N4, H9N2, H10N7, H10N9, H11N6, H12N5, H13N6, H14N6, H15N9, H16N3, APMV-1, APMV-2, APMV-3, APMV-4, APMV-6, APMV-7, APMV-8, and APMV-9 produced by Veterinary Laboratories Agency (Weybridge, UK) and the antisera H1N1, H2N3, H3N8, H4N8, H5N3, H6N2, H7N3, H8N4, H9N7, H10N1, H11N9, H12N5, H13N6, H14N5, H15N9, H16N3, APMV-1, APMV-2, APMV-3, APMV-4, APMV-6, APMV-7, APMV-8, and APMV-9 produced by the Istituto Zooprofilattico Sperimentale delle Venezie (Padova, Italy).

Sequencing. Gene sequence analysis was conducted on the H5 and N1 genes of the AIV isolates. Sequencing templates for individual influenza genes were produced by amplifying partial HA or NA genes with degenerate and end-specific primers by RT-PCR as previously described (12). Templates were then purified by agarose gel extraction with the QIAquick gel extraction kit (Qiagen, Valencia, CA). The BigDye terminator kit (Applied Biosystems, Foster City, CA) was used for direct cycle sequencing and subsequently run on an AB 3730 (Applied Biosystems).

Phylogenetic analysis. Phylogenetic analysis included the portion HA1 of the H5 gene from nucleotide (nt) 12 of the coding region to approximately nt 570 (GenBank accession numbers JX262650–JX262652). The portion of the N1 NA gene analyzed corresponded to nt 60 to nt 580 of the coding region (GenBank accession numbers JX262653–JX262657). Isolates representing other known major lineages were included in the phylogenetic reconstruction, and the Basic Local Alignment Search Tool search was used to identify the most closely related isolates. Sequences were aligned with Clustal V (Lasergene, v. 8.0.2; DNAStar, Madison, WI). Trees were constructed with BEAST v. 1.4.8 (4) using Hasegawa-Kishino-Yano substitution, empirical base frequency, gamma heterogeneity, codon 2 partitions, relaxed lognormal clock, Yule process tree prior with default operators with unweighted pair group mean with arithmetic average starting tree, and a Markov chain Monte Carlo length of 10^6.

RESULTS

From September 2010 to December 2011, 3857 samples were collected from 66 different species of wild birds in the Azov and Black Sea regions of Ukraine for virological examination. The wild bird species belonged to eight orders: Pelecaniformes, Ciconiiformes, Anseriformes, Galliformes, Gruiformes, Charadriiformes, Coraciiformes, and Passeriformes (Table 1). Of the 66 species, 46 represent the local avifauna breeding in the territory of the Azov and Black Sea

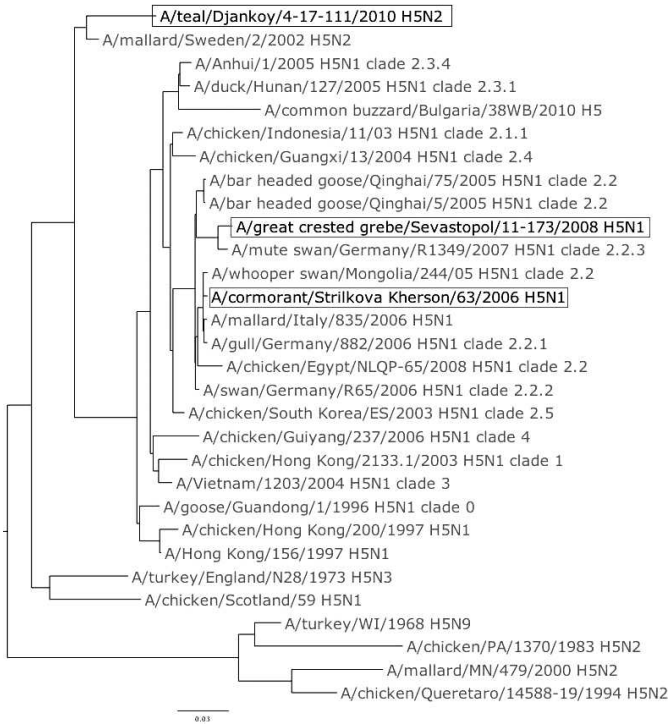

Fig. 2. Phylogenetic tree of the HA1 region of the H5 genes based on nucleotide sequence. Sequences from isolates collected in this study are shown in boldface.

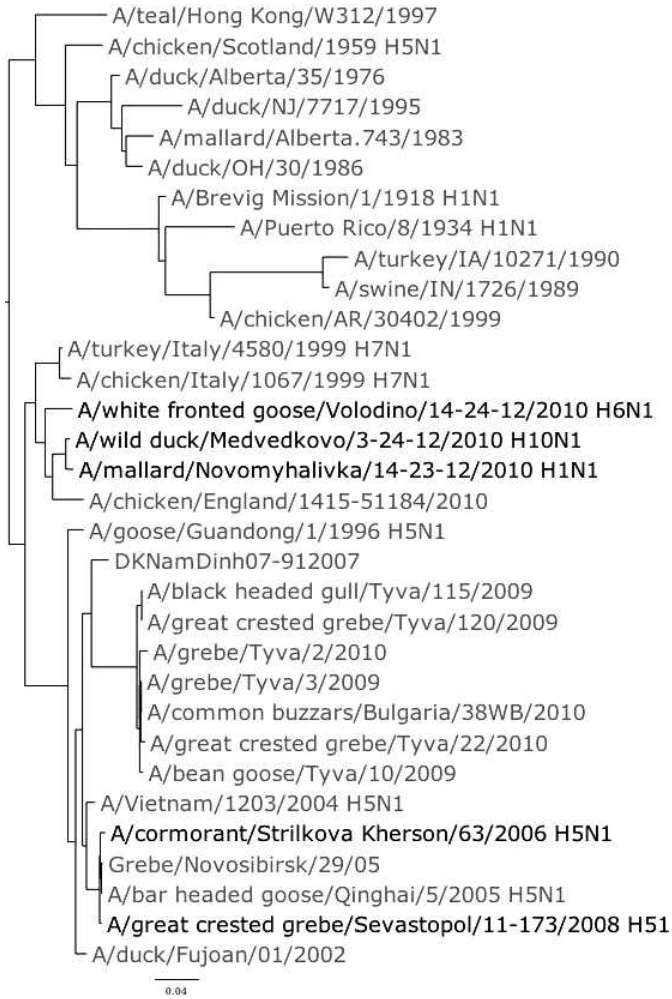

Fig. 3. Phylogenetic tree of the N1 genes based on nucleotide sequence. Sequences from isolates collected in this study are shown in boldface.

regions. Twenty species do not breed in the territory of study, stopping only during migration, including 11 short-range migrants from the northern territories of Ukraine, Belarus, Russia, and other neighboring areas and nine long-distance migrants from the Russian tundra, Southern Europe (probably the Volga Delta), and Kazakhstan. Most of the species represented in the samples in one way or another are connected with wetlands: 17 species of waterfowl and another 28 species belonging to wetland birds. All other birds are terrestrial species. Before beginning the study, a census of the number of birds in the projected areas of surveillance was conducted. By ornithologists' estimations, during October 2010 there were 421,711 birds belonging to 90 species in the Eastern Sivash (17). The most widespread bird species had a wetland habitat, particularly Anseriformes.

From September to November 2010, during the autumn migration, 962 samples were collected from 25 species of birds in the wetlands of Sivash (Kherson and Zaporizhia regions of Ukraine and Crimea), as well as from hunter-killed birds in Nizhnegorsky, Dzhankojsky, and Krasnoperekopskyi districts of Crimea (Fig. 1). During this period, 14 influenza viruses of different subtypes were isolated: H1N?, H3N8, H5N2, H7N?, H8N4, H10N7, and H11N8. For the H1N? and H7N? viruses we could not determine the subtype of the neuraminidase by serological tests because they did not react with any of the antisera. All of these viruses were isolated from waterfowl: shelduck (1), mallard (11), teal (1), and shoveler (1).

During the winter (December 2010–February 2011) the number of birds in the Azov and Black Sea regions of Ukraine changed. According to the ornithologists, in the wetlands of Sivash the total number of waterfowl (Anseriformes) was approximately 62,000 birds in December 2010, with approximately 28,000 additional birds belonging to other genera and other habitat groups. Seventy-six bird species were identified. When reevaluated in February 2011, the total number of wild birds in the Sivash decreased to 48,000, and the number of species was 73. During this period, 1966 samples were collected from 19 species of birds wintering in this region. These samples were collected in the Askania-Nova Reserve and in the Sivash wetlands (Fig. 1). Forty-five HA viruses were isolated from these samples. Thirty-six of them were AIVs of subtypes H1N1, H1N2, H1N?, H4N?, H6N1, H7N3, H7N6, H7N7, H8N2, H9N2, H10N7, H10N4, H11N2, H11N6, H12N2, and H15N7. We were not able determine the neuraminidase subtype for the H1N? and H4N? viruses with the available antisera. The AIVs were isolated from wild waterfowl samples: white-fronted goose (9), shelduck (7), ruddy shelduck (4), mallard (13) and yellow-legged gull (1). Two other AIVs were isolated from wild ducks whose species could not be identified because of bad weather.

The last set of samples were collected during the spring migration, nesting, and postnesting seasons (March to August 2011). This period is characterized by a lower density of wild birds in the areas surveyed. Spring migration was short, and wild bird density was low. During nesting, most birds did not form large clusters or colonies, especially waterfowl (geese and ducks). As during the postnesting, the concentration of birds depended on the species, which was reflected in the surveillance results. In total, during this period 706 samples were collected from 44 species of birds. Most of the

biological material collected during the spring migration and breeding were from the Askania-Nova Reserve and the Black Sea Biosphere Reserve. Samples from wild birds in the postbreeding movements were collected in wetlands in the Utlyuksky and Molochniy estuaries of the Velykyi Utluk River (Zaporizhia region). Only three HA viruses were isolated from the samples collected and were identified as AIV subtypes H6, H13, and H16 (NA not determined). These viruses were collected from two gull species and a wood sandpiper (*Tringa glareola*).

The remaining 14 HA viruses that were isolated between September 2010 and August 2011 were identified as different serotypes of avian paramyxoviruses: APMV-1, APMV-4, APMV-6, and APMV-7. Details of these isolates will be presented elsewhere.

DISCUSSION

In this study, 14 of the 16 (no H2 or H14) known HA subtypes of AIV were isolated from samples collected from wild birds from the Azov and Black Sea regions, indicating that AIVs circulate among different wild bird species in this area. Similarly all NA subtypes except N5 and N9 were isolated. The most common HA subtypes isolated were H1 (eight viruses; H1N1, H1N2), H6 (seven viruses; H6N1), H7 (seven viruses; H7N3, H7N6, H7N7), H11 (six viruses; H11N8, H11N2, H11N6), H3 (five viruses; H3N8), H8 (four viruses; H8N4, H8N2), H10 (four viruses; H10N7, H10N4), and H9 (three viruses; H9N2). Only one virus was isolated from each of the other HA subtypes (H5, H12, H13, H15, and H16).

Most of the viruses (49 isolates) were isolated from Anseriformes and only four viruses from Charadriiformes. No AIV was isolated from any of the other six orders sampled. For the different seasons, the virus prevalence, based on virus isolation, varied from 0.42% to 1.83%. The average prevalence for the whole year was 1.46%. The greatest number of AIVs were isolated during the winter (36 isolates; 1.83%), and during the autumn migration, when 14 AIVs were detected (1.45%). In contrast, only three AIVs (0.42%) were isolated from wild birds during spring migration, nesting, and postnesting movements.

These results on virus isolations may be related to geographic and climatic features of the Azov and Black Sea regions of Ukraine, which determine the abundance and diversity of bird species. Thus, according to ornithologists, the largest concentration of wild birds in a short period of time in a limited area, especially Anseriformes and Charadriiformes from different geographical regions of Siberia, Asia, and the northern part of Eurasia, happens during the autumn migration and wintering. This underlines the importance of this season and region in the ecology of viruses such as AIV and APMV. Studies like this can further our understanding of the circulation of viruses in wild birds and predict possible introductions of viruses into other geographic regions. As for the spring migration, nesting, and postnesting season, the number of birds is much lower. Therefore, in our opinion, this season is not as significant as the autumn and winter seasons for the spread of viruses.

The HA and NA genes of the H5 or N1 viruses from this study were sequenced as well as some earlier H5N1 viruses from the Institute's repository. The H5 HA genes clustered within two lineages (Fig. 2). One lineage included A/teal/Djankoy/40-17-11/2010, the most recent H5 isolate (H5N2), and was most closely related to wild bird LPAIVs found in wild birds in Europe. The other two H5 isolates collected earlier, A/great created grebe/Sevastopol/11-173/2008 (H5N1) and A/cormorant/Strilkova/Kherson/63/2006 (H5N1), were most closely related to the clade 2.2 Asian H5N1 HPAIV lineage that spread from the Qinghai Lake outbreak into Europe and Northern Asia (3). The N1 genes followed a similar pattern where the earlier H5N1 isolates, A/great created grebe/Sevastopol/11-173/2008 and A/cormorant/Strilkova/Kherson/63/2006, were closely related to the Qinghai Lake lineage. The N1 NAs from the more recent isolates, A/white fronted goose/Volodino/14-24-12/2010 H6N1, A/wild duck/Mevedkovo/3-24-12/2010 H10N1, and A/mallard/Novomyhalivka/14/-23-12/2010 H10N1, appear to be most closely related to LPAIVs from Europe (Fig. 3). This demonstrates that these regions of Ukraine are ecologically connected with Europe and underlines the importance of this region as an area of convergence of different flyways, also noted in a previous study (6).

Wild bird surveillance studies were previously conducted in Ukraine from 2006 to 2008 in the Crimea region (6). Compared with these studies, we identified a larger number of influenza virus subtypes H6, H7, H8, and H11 and a smaller number of H3 and H4 viruses. This confirms that even between years, different results might be obtained in the same region. It is therefore very important to continue the monitoring of wild birds at the intersection of migration routes. Similar to other wild bird surveillance studies, most of our viruses were isolated from Anseriformes and Charadriiformes, with the highest isolation rates from mallards. Isolation rates vary widely among wild bird surveillance studies, but the highest rates, which were observed in the autumn, were consistent with previous studies of waterfowl from North America (10). On the other hand, the lowest rates of virus isolation are typically seen in wintering birds in both Europe and North America, unlike what was observed in this study.

REFERENCES

1. Capua, I., and D. J. Alexander. Avian influenza and Newcastle Disease: a field and laboratory manual. Springer, New York. 2009.

2. Chen, H., G. D. J. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. M. Peiris, and Y. Guan. H5N1 virus outbreak in migratory waterfowl: a worrying development could help to spread this dangerous virus beyond its stronghold in southeast Asia. Nature 436:191–192. 2005.

3. Chen, H., Y. Li, Z. Li, J. Shi, K. Shinya, G. Deng, Q. Qi, G. Tian, S. Fan, H. Zhao, Y. Sun, and Y. Kawaoka. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J. Virol. 80:5976–5983. 2006.

4. Drummond, A. J., and A. Rambaut. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7:214. 2007.

5. [O.I.E.] World Organisation for Animal Health. Avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals 2011. World Organisation for Animal Health, Paris. Available from: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf. 2012.

6. Kulak, M. V., F. A. Ilinykh, A. V. Zaykovskaya, A. V. Epanchinzeva, I. L. Evstaphiev, N. N. Tovtunec, K. A. Sharshov, A. G. Durimanov, N. A. Penkovskaya, A. M. Shestopalov, A. I. Lerman, I. G. Drozdov, and D. E. Swayne. Surveillance and identification of influenza A viruses in wild aquatic birds in the Crimea, Ukraine (2006–2008). Avian Dis. 54:1086–1090.

7. Muzyka, D. V., B. T. Stegniy, V. O. Busol, A. N. Rula, K. F. Mayorova, and S. V. Homenko. Epizootic situation concerning highly-pathogenic avian influenza (H5N1) among wild birds in Ukraine. Vet. Med. 87:128–138. 2006.

8. Pedersen, J. C. Neuraminidase-inhibition assay for the identification of influenza A virus neuraminidase subtype of neuraminidase antibody specificity. In: Avian influenza viruses: methods in molecular biology. E. Spackman, ed. Humana Press, Totowa, NJ. pp. 67–75. 2008.

9. Stallknecht, D. E. Ecology and epidemiology of avian influenza viruses in wild bird populations: waterfowl, shorebirds, pelicans, cormorants, etc. Fouth International Symposium on Avian Influenza. 61–69. 1998.

10. Stallknecht, D. E., and J. D. Brown. Ecology of avian influenza in wild birds. In: Avian influenza. D. E. Swayne, ed. Blackwell, Ames, IA. pp. 43–58. 2008.

11. Stegniy, B. T., A. I. Buzun, D. V. Muzyka, M. V. Miroshnichenco, E. N. Belyavtseva, N. M. Pasyuga, and S. M. Kuzemko. Influenza of natural water reservoirs factors on the rise of highly pathogenic avian influenza in AR Crimea. Vet. Med. 87:186–192. 2006.

12. Suarez, D. L., M. Garcia, J. Latimer, D. Senne, and M. Perdue. Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. J. Virol. 73:3567–3573. 1999.

13. Swayne, D. E., and D. A. Halvorson. Influenza. In: Diseases of poultry, 12th ed. J. R. Glisson, Y. M. Saif, A. M. Fadly, L. R. McDougald, and L. Nolan, eds. Blackwell, Ames, Iowa. pp. 153–184. 2008.

14. Swayne, D. E., and M. Pantin-Jackwood. Pathobiology of avian influenza virus infections in birds and mammals. In: Avian influenza. D. E. Swayne, ed. Blackwell Publishing, Ames, IA. pp. 87–122. 2008.

15. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56:152–179. 1992.

16. Stegniy, B. T., I. Yu. Bisuk, A. P. Gerilovych, A. I. Buzun, and D. V. Muzyka. Analysis of epizootic situation concerning highly pathogenic avian influenza areas on the territory of the AR Crimea, isolation and identification of the agent. Vet. Med. Ukraine 1:14–17. 2007.

17. Chernichko, I. I., and V. A. Kostyushin, eds. Results of the regional ornithological monitoring (Azov–Black Sea coast of Ukraine). Bulletin ROM 6 (October). 24 p. 2010.

ACKNOWLEDGMENTS

The authors express their sincere thanks to ornithologists R. M. Chernichko, I. I. Chernichko, Y. A. Andryushchenko, and V. M. Popenko for help in catching birds and identifying bird species as well as providing relevant information. This research was funded by USDA Project P444 through the Science and Technology Center in Ukraine (STCU).