The H5 highly pathogenic avian influenza viruses (HPAIVs) of clade 2.3.4.4 are of great concern because of their global spread and circulation. Ample evidence indicates that clade 2.3.4.4 H5 viruses derived neuraminidase (NA) gene from other low-pathogenicity avian influenza viruses (LPAIVs) co-circulating in migratory birds, and new subtypes of H5N2, H5N5, H5N6, and H5N8 HPAIVs have been detected in wild bird species and poultry globally (1,2). To date, H5 viruses of clade 2.3.4.4 have evolved into 8 subclades (2.3.4.4a to 2.3.4.4h) according to the World Health Organization’s (WHO) nomenclature system (1). Among them, H5N6 is the only subtype that has caused human infections. As of August 2019, a total of 24 human cases have been reported to WHO; the mortality rate is 67% (3,4).

H5N6 virus of subclade 2.3.4.4a was first detected in poultry in Laos in 2013, then spread to Vietnam and China and caused numerous cases in these areas. H5N8 virus of subclade 2.3.4.4b caused disease outbreaks in wild birds and poultry in Korea in 2014, then spread to North America through bird migration and established a new subclade, 2.3.2.4c. When the H5N8 virus of subclade 2.3.4.4b landed in Europe and Africa, it reassorted with the local LPAIV and produced H5N6 with a novel internal gene cassette in 2017 (5). Simultaneously, the H5N6 viruses of subclades 2.3.4.4d, 2.3.4.4e, 2.3.4.4f, 2.3.4.4g, and 2.3.4.4h established in poultry and wild birds in Southeast Asia (1,6–8). Among the 8 subclades of 2.3.4.4, only 3 (H5N6 2.3.4.4b, 2.3.4.4e, and 2.3.4.4f) had been previously detected in swans (1).

Since 2004, different vaccines have been developed and widely administered among poultry flocks in China and other countries for H5 avian influenza control, and the vaccine seed viruses used in China have been updated regularly to ensure antigenic match between the vaccine strain and the prevalent strains (9,10). After the H7N9 HPAIVs emerged in China in 2017, an H5/H7 combined inactivated vaccine was developed and used in poultry (11,12). Currently, the vaccine seed virus Re-11 is being used to control the clade 2.3.4.4 viruses (10). In our study, we analyzed the genetic evolution, antigenicity, and pathogenicity of the H5N6 HPAIVs isolated from migratory whooper swans (Cygnus cygnus) and mute swans (C. olor) in Xinjiang, western China, in January 2020.

The Study
The first sick whooper swan was found on December 29, 2019, in Sala Village, Samuyuzi Township, Yinling City, Xinjiang Uyghur Autonomous Region. The bird died on January 1, 2020. By January 17, deaths had been reported in 58 swans in 6 locations (Table 1; Figure 1, panel A). We received 5 batches of clinical
samples from 13 dead birds (11 whooper swans and 2 mute swans), and 13 H5N6 viruses were isolated. The hemagglutinin (HA) subtypes were identified by a hemagglutinin-inhibition test with a panel of H1–H16 subtype antisera, whereas the NA subtypes were detected by reverse transcription PCR with a panel of N1–N9 subtype-specific primers (11).

To trace the origin of the viruses and understand their genetic relationship, we sequenced the genome of the 13 viruses and performed comparative phylogenetic analysis with the representative H5 HPAIVs that were recommended by WHO (1). All 13 H5N6 viruses possess high identity with each other (99.5%–100%); 7 of 8 segments are closely related to the H5N6 virus isolated from environmental samples in Guangdong Province in 2017, whereas the other 1, nonstructural protein gene, is closest to A/chicken/Ngh An/01VTC/2018(H5N6) (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/26/12/20-1201-App1.pdf). The HA gene has the typical highly pathogenic amino acid sequence -RRKR- in its cleavage site, and a few mammalian adaptation mutations were detected in the genome (Table 2) (13). In the maximum clade credibility tree, the HA genes of the 13 H5N6 viruses are grouped into subclade 2.3.4.4h with the HA genes of the strains recently found in Vietnam, China, and Russia (1) (Figure 1, panel B). The neighbor-joining phylogenetic trees of the 8 gene segments are shown in Appendix Figure 1.

The hemagglutinin-inhibition test was performed with polyclonal antiserum generated from the SW/XJ/1/2020(H5N6) and the currently used H5N1
DISPATCHES

inactivated vaccine Re-11, which carries the HA gene from A/duck/Guizhou/S4184/2017(H5N6) virus (10). We found that the SW/XJ/1/2020(H5N6) cross-reacted well with Re-11 antisera, and vice versa (Appendix Table 2), yielding a cross-reactivity R value of 0.26.

We conducted an intravenous pathogenicity index test in chickens with the index virus, WS/XJ/1/2020(H5N6), by following the protocol of the World Organisation for Animal Health (OIE) (14). Ten 6-week-old specific-pathogen–free chickens were inoculated with 0.2 mL of virus intravenously, and all the birds died within 3 days postinoculation, yielding an intravenous pathogenicity index test value of 2.59.

We tested the virulence and transmission of the WS/XJ/1/2020(H5N6) in ducks as previously described (2). Eight 3-week-old specific-pathogen–free ducks were intranasally inoculated with $10^6$ 50% egg infective dose (EID$_{50}$) WS/XJ/1/2020(H5N6), and 3 uninfected ducks were put in the same cage 24 hours later for monitoring transmission. Three virus-inoculated ducks were euthanized on day 3 postinoculation, and high titers of virus were detected in the tested organs (Figure 2, panel A). Virus was also detected in the oropharyngeal and cloacal swabs of the surviving virus-inoculated ducks and the contact ducks on days 3 and 5 postinoculation (Figure 2, panel B). All

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acid/motif</th>
<th>Phenotypic consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemagglutinin</td>
<td>Cleavage motif: -RRKR G-</td>
<td>Polybasic cleavage motif sequence required for high pathogenicity of avian influenza viruses in chickens</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>Stalk deletion 58–68</td>
<td>Increased virulence in mice</td>
</tr>
<tr>
<td>Polymerase acidic protein</td>
<td>515T</td>
<td>Increased polymerase activity in mammalian cells</td>
</tr>
<tr>
<td>Matrix protein 1</td>
<td>30D</td>
<td>Increased virulence in mice</td>
</tr>
<tr>
<td></td>
<td>215A</td>
<td>Increased virulence in mice</td>
</tr>
<tr>
<td>Nonstructural protein 1</td>
<td>80–84 deletion</td>
<td>Increased virulence in mice</td>
</tr>
<tr>
<td></td>
<td>42S</td>
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<tr>
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<td>98F</td>
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<td>101M</td>
<td>Increased virulence in mice</td>
</tr>
<tr>
<td></td>
<td>222–225 ESEV (PDZ domain)</td>
<td>Increased virulence in mice</td>
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Figure 2. Replication and virulence of the WS/XJ/1/2020(H5N6) isolate in ducks and mice in a laboratory test performed after H5N6 avian influenza (H5N6) outbreaks among migratory whooper swans (Cygnus cygnus), Xinjiang Province, China, January 2020. A) Viral titer in organs of ducks that were euthanized on day 3 postinoculation. B) Viral titers in oropharyngeal and cloacal swabs from all surviving ducks were collected on days 3 and 5 postinoculation. C) Lethality of the virus in ducks. D) Viral titer in organs of mice that were euthanized on day 3 postinoculation. E) Bodyweight change of mice after inoculation with different doses of the virus. F) MLD$_{50}$ of the virus. Viral titers in panels A, B, and D are shown as the mean $\pm$ SD. The dashed lines indicate the lower limit of detection. EID$_{50}$, 50% egg infective dose; MLD$_{50}$, 50% mouse lethal dose.
5 virus-inoculated ducks and 3 contact ducks died within 7 days postinoculation (Figure 2, panel C).

The replication and 50% mouse lethal dose (MLD$_{50}$) of the WS/XJ/1/2020(H5N6) were evaluated in BALB/c mice as previously reported (2). Three mice were intranasally inoculated with 10$^6$ EID$_{50}$ of WS/XJ/1/2020(H5N6) in a volume of 50 µL and were euthanized on day 3 postinoculation to assess virus replication in organs, and we found the virus in the brain of 1 mouse, the spleens of 2 mice, and the nasal turbinates and lungs of all 3 mice, but not in the kidneys of any mouse (Figure 2, panel D). To test the MLD$_{50}$ groups of five 6-week-old mice were intranasally inoculated with 10$^1$ to 10$^6$ EID$_{50}$ of WS/XJ/1/2020(H5N6) in a volume of 50 µL and were monitored for bodyweight loss and death for 14 days. Only 1 of 5 mice that received the highest dose of 10$^6$ EID$_{50}$ died on day 8 postinoculation; all other mice survived the 14-day observation period, yielding an MLD$_{50}$ value of 6.38 log$_{10}$ EID$_{50}$ (Figure 2, panel E, F).

**Conclusions**

A total of 58 swans died from H5N6 virus infection in 6 wild bird habitats in Xinjiang in January 2020, and we isolated 13 similar H5N6 HPAIVs from the swan specimens. These viruses bear the HAs of subclade 2.3.4.4h, which were previously detected in other bird species but not in swans.

The WS/XJ/1/2020(H5N6) is highly pathogenic to chickens and ducks, and antigenically close to the H5N1 vaccine seed virus Re-11. Although the virus is low pathogenic in mice, it bears multiple residues that can increase its virulence in mammals, and thus might pose a potential threat to public health.

Wild birds carry and spread the H5 HPAIV, as evidenced by the dissemination of the clade 2.2 viruses from Asia to Europe and Africa in 2005, and the intercontinental distribution of the clade 2.3.4.4b viruses in 2014 (5,15). The prospect of these H5N6 viruses detected in swans being distributed widely by wild birds is worrisome. Therefore, with the migratory season coming, surveillance and preventive measures should be implemented in poultry raised on the migration routes of wild birds.

**Acknowledgments**

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October 2020

Bacterial Infections

• Operating Protocols of a Community Treatment Center for Isolation of Patients with Coronavirus Disease, South Korea
• Community Treatment Centers for Isolation of Asymptomatic and Mildly Symptomatic Patients with Coronavirus Disease, South Korea
• Clinical Course of Asymptomatic and Mildly Symptomatic Patients with Coronavirus Disease Admitted to Community Treatment Centers
• Nationwide External Quality Assessment of SARS-CoV-2 Molecular Testing, South Korea
• Impact of Social Distancing Measures on Coronavirus Disease Healthcare Demand, Central Texas, USA
• Multicenter Prevalence Study Comparing Molecular and Toxin Assays for Clostridioides difficile Surveillance, Switzerland
• Effectiveness of 23-Valent Pneumococcal Polysaccharide Vaccine against Invasive Pneumococcal Disease in Adults, Japan, 2013–2017
• Sequential Acquisition of Human Papillomavirus Infection at Genital and Anal Sites, Luizhou, China
• Association between Shiga Toxin–Producing Escherichia coli O157:H7 stx Gene Subtype and Disease Severity, England, 2009–2019
• Rapid, Sensitive, Full-Genome Sequencing of Severe Acute Respiratory Syndrome Coronavirus 2
• Effect of Nonpharmaceutical Interventions on Transmission of Severe Acute Respiratory Syndrome Coronavirus 2, South Korea, 2020
• Main Routes of Entry and Genomic Diversity of SARS-CoV-2, Uganda
• High Proportion of Asymptomatic SARS-CoV-2 Infections in 9 Long-Term Care Facilities, Pasadena, California, USA, April 2020
• Tickborne Relapsing Fever, Jerusalem, Israel, 2004–2018
• Seawater-Associated Highly Pathogenic Francisella hispaniensis Infections Causing Multiple Organ Failure

• Basic Reproduction Number of Chikungunya Virus Transmitted by Aedes Mosquitoes
• Deaths Associated with Pneumonic Plague, 1946–2017
• Emerging Sand Fly-Borne Phlebovirus in China
• Drug Resistance Spread in 6 Metropolitan Regions, Germany, 2001–2018
• Human Adenovirus B7–Associated Urethritis after Suspected Sexual Transmission, Japan
• Polyester Vascular Graft Material and Risk for Intracavitary Thoracic Vascular Graft Infection
• Silent Circulation of Rift Valley Fever in Humans, Botswana, 2013–2014
• Limitations of Ribotyping as Genotyping Method for Corynebacterium ulcerans
• Seoul Orthohantavirus in Wild Black Rats, Senegal, 2012–2013
• Contact Tracing during Coronavirus Disease Outbreak, South Korea, 2020
• Pooling Upper Respiratory Specimens for Rapid Mass Screening of COVID-19 by Real-Time RT-PCR
• Coronavirus Disease among Persons with Sickle Cell Disease, United States, March 20–May 21, 2020
• Eliminating Spiked Bovine Spongiform Encephalopathy Agent Activity from Heparin

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