Characterization of Highly Pathogenic Avian Influenza Virus A(H5N6), Japan, November 2016

Masatoshi Okamatsu,1 Makoto Ozawa,1 Kosuke Soda,1 Hiroki Takakuwa,1 Atsushi Haga, Takahiro Hiono, Aya Matsuu, Yuko Uchida, Ritsuko Iwata, Keita Matsuno, Masakazu Kuwahara, Toshiyo Yabuta, Tatsufumi Usui, Hiroshi Ito, Manabu Onuma, Yoshihiro Sakoda, Takehiko Saito, Koichi Otsuki, Hiroshi Ito, Hiroshi Kida

Highly pathogenic avian influenza viruses (HPAIVs) A(H5N6) were concurrently introduced into several distant regions of Japan in November 2016. These viruses were classified into the genetic clade 2.3.4.4c and were genetically closely related to H5N6 HPAIVs recently isolated in South Korea and China. In addition, these HPAIVs showed further antigenic drift.

Since their emergence in ≈2010–11 in China (1), highly pathogenic avian influenza viruses (HPAIVs) that have the hemagglutinin (HA) genes of the H5 subtype classified into the genetic clade 2.3.4.4 have threatened global bird species, including wild birds and poultry, as well as humans. Although the H5 HA genes of these viruses are closely related, the subtypes of their neuraminidase (NA) genes vary widely. These new H5 HPAIVs with NA genes of various subtypes, the so-called H5Nx viruses, have spread globally, most likely because of their host preference for waterfowl, similar to the previous H5N1 HPAIVs (2–4). During the winter season 2014–15, H5N8 HPAIVs were isolated from wild birds and chickens in western Japan (5–7). In November 2016, HPAIVs of the H5N6 subtype were isolated in 3 geographically distant regions of Japan. We report the genetic and antigenic characteristics of 6 H5N6 HPAIVs.

The Study
The first suspected case of an HPAI outbreak in Japan during winter 2016–17 was reported from Akita Prefecture in northern Japan (Figure 1). A black swan (Cygnus atratus) in a zoo that died on November 15, 2016, tested positive for influenza virus antigen by a rapid diagnostic test. While this bird’s specimens underwent further analysis, another influenza virus was isolated from a water sample collected at an overwintering site of migratory birds in Kagoshima Prefecture at the southern tip of Japan on November 14, 2016 (Table 1). This isolate, A/environment/Kagoshima/KU-ngr-I/2016 (H5N6), was confirmed to be an H5N6 subtype having multiple basic amino acid residues, PLRERRRKR/GLF, at the cleavage site in the HA protein, which is characteristic of HPAIVs, by conventional reverse transcription PCR and Sanger sequencing. Subsequently, an isolate from the first black swan, A/black swan/Akita/1/2016 (H5N6), also was confirmed to be an H5N6 HPAIV, showing that all 3 chickens inoculated intranasally with 10⁸.4 of 50% egg infectious dose of the virus died within 2 days. In addition, a fecal sample of a common teal (Anas crecca) collected at an overwintering site of migratory birds in Tottori Prefecture in the middle of Japan on November 15, 2016, was reported to harbor an H5N6 HPAIV, A/teal/Tottori/b37/2016 (H5N6), also confirmed to be an H5N6 HPAIV, showing that all 3 chickens inoculated intranasally with 10⁸.4 of 50% egg infectious dose of the virus died within 2 days. In addition, a fecal sample of a common teal (Anas crecca) collected at an overwintering site of migratory birds in Tottori Prefecture in the middle of Japan on November 15, 2016, was reported to harbor an H5N6 HPAIV, A/teal/Tottori/b37/2016 (H5N6), and A/crane/Kagoshima/KU-4/2016 (H5N6), in Japan (Table 1). As of December 4, 2016, a total of 31 confirmed cases in wild birds had been reported to the Ministry of Environment (http://www.env.go.jp/nature/dobutsu/bird_flu/index.html), and 4 cases at poultry farms were confirmed in Japan (8).

To clarify the genetic background of the H5N6 HPAIVs concurrently introduced into several distant regions of Japan, we determined the complete genome sequences of 5 of our isolates: A/black swan/Akita/2/2016 (H5N6), A/northern pintail/Tottori/337/2016 (H5N6), and A/crane/Kagoshima/KU-4/2016 (H5N6), in Japan (Table 1). As of December 4, 2016, a total of 31 confirmed cases in wild birds had been reported to the Ministry of Environment (http://www.env.go.jp/nature/dobutsu/bird_flu/index.html), and 4 cases at poultry farms were confirmed in Japan (8).

Characterization of Highly Pathogenic Avian Influenza Virus A(H5N6), Japan, November 2016

Author affiliations: Hokkaido University, Sapporo Japan (M. Okamatsu, T. Hiono, K. Matsuno, Y. Sakoda, H. Kida); Kagoshima University, Kagoshima, Japan (M. Ozawa, A. Matsuu); Yamaguchi University, Yamaguchi, Japan (M. Ozawa, A. Matsuu); Tottori University, Tottori, Japan (K. Soda, T. Usui, H. Ito, K. Otsuki, T. Ito); Kyoto Sangyo University, Kyoto, Japan (H. Takakuwa, Y. Yabuta, K. Otsuki); Matsuoka Research Institute for Science, Koganei, Tokyo, Japan (M. Kuwahara); National Agriculture and Food Research Organization, Tsukuba, Japan (Y. Uchida, T. Saito); National Institute for Environmental Studies, Tsukuba (A. Haga, R. Iwata, M. Onuma)

DOI: http://dx.doi.org/10.3201/eid2304.161957

These authors contributed equally to this article.
DISPATCHES

nos. LC198525–LC198532), A/teal/Tottori/1/2016 (H5N6) (GenBank/DDBJ/EMBL accession nos. LC198965–LC199872), A/northern pintail/Tottori/b37/2016 (H5N6) (GenBank/DDBJ/EMBL accession nos. LC200414–LC200421), A/environment/Kagoshima/KU-ngr-I/2016 (H5N6) (GISAID EpiFlu [http://platform.gisaid.org/], GenBank/DDBJ/EMBL accession nos. EPI861582–EPI861589), and A/crane/Kagoshima/KU-4/2016 (H5N6) (GenBank/DDBJ/EMBL accession nos. EPI867577–EPI867584) by Sanger and/or Illumina Miseq next-gen-eration sequencing. These 5 isolates were almost genetically identical. Even among the HA genes, which are the most frequently mutated ones among the 8 gene segments, only 3–8 nt mutations, including 3 nonsynonymous mutations, were detected compared with the earliest strain, A/northern pintail/Tottori/b37/2016 (H5N6) (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/4/16-1957-Techapp1.pdf). Thus, the 5 isolates would share a close common ancestor.

The phylogenetic tree analysis of the HA gene revealed that our isolates are classified into the genetic clade 2.3.4.4c and clustered with the recent H5N6 HPAIV isolates from wild and domestic birds and humans in China, in addition to an isolate South Korea, A/Mandarin duck/Korea/K16-187-3/2016 (H5N6) (Figure 2, panel A), on the basis of a recent classification in clade 2.3.4.4 (9,10). The NA genes of our isolates also form a single cluster together with the H5N6 HPAIV isolates from China into group C in the phylogenetic tree (Figure 2, panel B). In addition, the remaining 6 genes were genetically close to the recent H5N6 HPAIV isolates from China in the corresponding phylogenetic trees (online Technical Appendix Figure 1), except for the polymerase basic 1 genes, which are most closely related to the counterpart of A/duck/Guangdong/S4040/2011 (H4N2) that was isolated from a domestic duck at a live bird market in China (11). Thus, the H5N6 HPAIV isolates would be derived from a reassortant that arose between an H5N6 HPAIV recently circulating in wild birds, poultry, or both in East Asia and in low pathogenicity avian influenza virus circulating in poultry in China. The genetic background of the H5N6 HPAIV isolates in this study is similar to the recent South Korea H5N6 virus collected in October 2016 and clearly different from that of recent H5Nx HPAIVs in Russia (10), Western European countries, and Alaska (8).

Our putative amino acid sequence comparison revealed that a leucine residue at position 134 in the HA protein (H3 numbering) was deleted, unlike that with the closest relative A/feline/Guangdong/1/2015 (H5N6) (online Technical Appendix Figure 2). Our isolates have the amino acid sequence QQG at positions 226–228, which are located at the receptor-binding site in the HA protein, although the corresponding amino acid sequences of the previous H5 viruses are QSG or QRG (online Technical Appendix Figure 2). These findings suggest that the receptor specificity of our H5N6 HPAIV isolates might be altered from their parental viruses (12,13). We also found 11 aa deletions in the stalk region of the NA protein, unlike that of A/duck/Vietnam/HU1–1151/2014 (H5N6), a representative virus strain of an N6 NA gene-based group D (online Technical Appendix Figure 3), which belongs to a different cluster of the clade 2.3.4.4.

For HA antigenic characterization, we investigated the reactivity of chicken antiserum raised against several H5 isolates to our H5N6 HPAIV isolates using the hemagglutination inhibition test (14). We selected 1 reference virus strain, A/environment/Kagoshima/KU-ngr-I/2016 (H5N6), to evaluate the reactivity. Table 1 presents the details of the isolates used.

Figure 1. Locations of confirmed highly pathogenic avian influenza virus A(H5N6) infections in Akita, Tottori, and Kagoshima Prefectures, Japan, 2016.
A/black swan/Akita/1/2016 (H5N6), and prepared single immunized chicken antiserum against the virus because of the limited variation of the nucleotide sequences in the HA genes among our 6 H5N6 HPAIV isolates. Antibody titer of antiserum of A/black swan/Akita/1/2016 (H5N6) were 16–32-fold higher against homologous virus than against the other strains (Table 2). The reactivity of the antiserum of A/chicken/Kumamoto/1-7/2014 (H5N8), whose HA gene belongs to the genetic clade 2.3.4.4, to A/black swan/Akita/1/2016 (H5N6) was 4-fold lower than that of the antiserum to the homologous combination. Moreover, none of the antiserum samples tested reacted strongly with A/black swan/Akita/1/2016 (H5N6) except for the homologous antiserum. These results indicate that the HA antigenicity of the H5N6 HPAIVs recently introduced in Japan differ appreciably from those of the previous H5Nx viruses.

**Conclusions**

We isolated 6 H5N6 HPAIVs from dead birds, fecal samples of migratory birds, and environmental water sample in 3 distant regions of Japan in November 2016. A genetic analysis showed that these isolates were genetically closely related to H5N6 HPAIVs recently isolated in China except for the polymerase basic 1 gene segment. The HA antigenicity of our H5N6 HPAIVs was demonstrated to have drifted further than viruses belonging to the same genetic clade 2.3.4.4. To prevent the spread of HPAIVs by wild birds, prompt elimination of HPAIVs is urgently needed in countries in Asia.
**DISPATCHES**

**Table 2. Antigenic analyses of H5 influenza viruses with antiserum**¹

<table>
<thead>
<tr>
<th>Virus lineage/clade</th>
<th>Virus</th>
<th>Hemagglutination inhibition titer of the antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mal/Hok</td>
<td>Wa/Hok</td>
</tr>
<tr>
<td>Eurasian</td>
<td>H5N1</td>
<td>H5N1</td>
</tr>
<tr>
<td>–</td>
<td>1,280</td>
<td>80</td>
</tr>
<tr>
<td>2.3.2.1</td>
<td>40</td>
<td>640</td>
</tr>
<tr>
<td>2.3.4</td>
<td>&lt;20</td>
<td>20</td>
</tr>
<tr>
<td>2.3.4.4 icA</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>2.3.4.4ic</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>2.5</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>North American</td>
<td>Ck/Ibaraki</td>
<td>320</td>
</tr>
</tbody>
</table>

¹Dash indicates virus does not belong to clade classification. Bold indicates virus isolated in this study. Underline indicates homologous titers. B, black swan; Ck, chicken; HK, Hong Kong; Hok, Hokkaido; Mal, mallard; Pf, peregrine falcon; Ws, whooper swan.

**Acknowledgments**

We thank Mizuho Suzuki, Aiko Ohnuma, Junki Maruyama, Ayato Takada, Satoshi Saito, Hiroichi Ozaki, Toshiyuki Murase, Tsuyoshi Yamaguchi, Natsuko Nishi, Kaori Totorozaki, Shigehisa Toda, Satoru Taura, Kotaro Kawabe, Atsushi Nishitani, and Naoko Maruta for their technical assistance. We thank the Ministry of the Environment, the Prefecture of Akita, the Prefecture of Tottori, and the City of Izumi for their kind cooperation.

This work was supported by a grant from the Project of the National Agriculture and Food Research Organization Bio-oriented Technology Research Advancement Institution (Integration Research for Agriculture and Interdisciplinary Fields) and by a grant for contracted research activity for crane conservation with the City of Izumi, Japan. This research was partially supported by the Japan Initiative for Global Research Network on Infectious Diseases from Japan’s Ministry of Education, Culture, Sport, Science and Technology, and Japan Agency for Medical Research and Development and by grants for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Science, Sports and Technology of Japan (JP16H06431, JP16H06429, and JP16K21723). This research was commissioned by the Kagoshima Crane Conservation Committee.

Dr. Okamatsu is an associate professor at the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan. His primary research interests are interspecies transmission and pathogenicity of influenza viruses.

**References**


Address for correspondence: Yoshihiro Sakoda, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, North 18, West 9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan; email: sakoda@vetmed.hokudai.ac.jp

October 2016: Disease Patterns

- Whole-Genome Characterization of Epidemic Neisseria meningitidis Serogroup C and Resurgence of Serogroup W in Niger, 2015
- Ebola Virus Disease in Children, Sierra Leone, 2014–2015
- Systematic Review and Meta-Analysis of the Treatment Efficacy of Doxycycline for Rectal Lymphogranuloma Venereum in Men who have Sex with Men
- Increase in Meningococcal Serogroup W Disease, Victoria, Australia, 2013–2015
- Distinct Zika Virus Lineage in Salvador, Bahia, Brazil
- Streptococcus suis Serotype 2 Capsule In Vivo
- Estimation of Severe MERS-CoV Cases in the Middle East, 2012–2016
- Hypervirulent Clone of Group B Streptococcus Serotype III Sequence Type 283, Hong Kong, 1993–2012
- Chikungunya Virus in Febrile Humans and Aedes aegypti Mosquitoes, Yucatan, Mexico

- Outbreaks of Human Salmonella Infections Associated with Live Poultry, USA, 1990–2014
- Vaccine-Derived Polioviruses and Children with Primary Immunodeficiency, Iran, 1995–2014
- Infection-Related Deaths from Refractory Juvenile Idiopathic Arthritis
- Accuracy of Diagnosis of Human Granulocytic Anaplasmosis in China
- Population-Level Effects of Human Papillomavirus Vaccination Programs on Infection with Nonvaccine Human Papillomavirus Genotypes
- Cat-Scratch Disease in the United States, 2005–2013
- Community- and Healthcare-Associated Clostridium difficile Infections, Finland, 2008–2013
- Carbapenem Resistance in Clonally Distinct Clinical Strains of Vibrio fluvialis Isolated from Diarrheal Samples
- Viral RNA in Blood as Indicator of Severe Outcome in Middle East Respiratory Syndrome Coronavirus Infection
- Sporotrichosis-Associated Hospitalizations, United States, 2000–2013
- Effect of Geography on the Analysis of Coccidioidomycosis-Associated Deaths, United States
- Novel Single-Stranded DNA Circular Viruses in Pericardial Fluid of Patient with Recurrent Pericarditis
- Unmet Needs for a Rapid Diagnosis of Chikungunya Virus Infection
- African Tick-Bite Fever in Traveler Returning to Slovenia from Uganda
- Synovial Tissue Infection with Burkholderia fungorum

http://wwwnc.cdc.gov/eid/articles/issue/22/10/table-of-contents