Jpn. J. Infect. Dis., 75, 398-402, 2022

Short Communication

Antiviral Susceptibilities of Avian Influenza A(H5), A(H7), and A(H9) Viruses Isolated in Japan

Emi Takashita[1]*, Hiroko Morita[1], Shiho Nagata[1], Masayuki Shirakura[1], Seiichiro Fujisaki[1], Hideka Miura[1], Ikuyo Takayama[1], Tomoko Arita[1], Yasushi Suzuki[1], Masaoki Yamaoka[2], Taichiro Tanikawa[3], Ryota Tsunekuni[4], Junki Mine[4], Saki Sakuma[4], Yuko Uchida[4], Akihiro Shibata[5], Mari Iwanaka[5], Noriko Kishida[1], Kazuya Nakamura[1], Tsutomu Kageyama[1], Shinji Watanabe[1], Hideki Hasegawa[1], and The Influenza Virus Surveillance Group of Japan[§]

[1]Research Center for Influenza and Respiratory Viruses, National Institute of Infectious Diseases, Tokyo;
[2]Hyogo Prefectural Institute of Public Health Science, Hyogo;
[3]Virus group, Division of Infectious Animal Disease Research and
[4]Emerging Virus Group, Division of Zoonosis Research, National Institute of Animal Health, National Agriculture and Food Research Organization, Ibaraki; and
[5]Animal Quarantine Service, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan

ABSTRACT: The circulation of avian influenza A viruses in poultry is a public health concern due to the potential transmissibility and severity of these viral infections. Monitoring the susceptibility of these viruses to antivirals is important for developing measures to strengthen the level of preparedness against influenza pandemics. However, drug susceptibility information on these viruses is limited. Here, we determined the susceptibilities of avian influenza A(H5N1), A(H5N2), A(H5N8), A(H7N7), A(H7N9), A(H9N1), and A(H9N2) viruses isolated in Japan to the antivirals approved for use there: an M2 inhibitor (amantadine), neuraminidase inhibitors (oseltamivir, peramivir, zanamivir, and laninamivir) and RNA polymerase inhibitors (baloxavir and favipiravir). Genotypic methods that detect amino acid substitutions associated with antiviral resistance and phenotypic methods that assess phenotypic viral susceptibility to drugs have revealed that these avian influenza A viruses are susceptible to neuraminidase and RNA polymerase inhibitors. These results suggest that neuraminidase and RNA polymerase inhibitors currently approved in Japan could be a treatment option against influenza A virus infections in humans.

The circulation of avian influenza A viruses in poultry is a public health concern because these viruses may become more transmissible and cause severe disease in humans. In 1997, during a highly pathogenic avian influenza A(H5N1) virus outbreak in poultry in Hong Kong, the highly pathogenic virus also infected humans (1). Furthermore, since 2003, this virus has spread and become endemic to poultry in some countries. A(H5N1) virus outbreaks have resulted in millions of poultry infections. From January 2003 to November 2021, 863 cases of human infection with the A(H5N1) virus were reported across 18 countries. Of the 863 cases, 456 were fatal. In 2013, human infections with avian influenza A(H7N9) virus were reported in China (2). Since then, this virus has spread to poultry throughout the country, and a total of 1,568 human infections, including 616 fatal cases, have been reported.

Other avian influenza A viruses have been detected sporadically in humans. The most frequent subtypes that cause human infections are A(H5), A(H7), and A(H9) viruses, and the World Health Organization (WHO) has assessed the public health risks of these viruses and coordinated the development of candidate vaccines for these subtypes (3). In Japan, avian influenza A(H5) and A(H7) virus outbreaks have been reported in poultry (OIE World Animal Health Information System: OIE-WAHIS, https://wahis.oie.int). Other avian influenza A viruses, including the A(H9) subtype, have been detected sporadically in poultry and wild birds through surveillance. Furthermore, avian influenza A(H5N1), A(H5N6), A(H7N9), and A(H9N2) viruses have been detected in raw poultry meat which was illegally

Received October 26, 2021. Accepted December 8, 2021.
J-STAGE Advance Publication December 28, 2021.
DOI: 10.7883/yoken.JJID.2021.751
*Corresponding author: Mailing address: Reseach Center for Influenza and Respiratory Viruses, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan. Tel: +81-42-848-7164, E-mail: emitaka@niid.go.jp
[§]The members of the group are listed in the Appendix.

brought by international flight passengers, confiscated, and stored in quarantine sections (4,5). However, no human cases have been reported to date in Japan.

Three classes of antivirals have been approved for influenza treatment or prophylaxis in Japan: M2 inhibitor (amantadine), neuraminidase (NA) inhibitors (oseltamivir, peramivir, zanamivir, and laninamivir), and RNA polymerase inhibitors (baloxavir and favipiravir). Since most avian influenza A(H5N1) and A(H7N9) viruses possess an S31N amino acid substitution in the M2 protein, which confers resistance to the M2 inhibitor, WHO does not recommend the use of M2 inhibitors for the treatment of these viral infections. Laninamivir, baloxavir, and favipiravir were developed and approved first in Japan. We previously reported NA inhibitor and favipiravir susceptibilities of avian influenza A(H7N9) viruses isolated from humans in China (6,7). However, information regarding the susceptibility of avian influenza A viruses to these three drugs is limited. Here, we determined the susceptibilities of avian influenza A(H5), A(H7), and A(H9) viruses isolated in Japan to the approved antivirals.

First, we examined representative avian influenza A(H5N1), A(H5N2), A(H7N7), A(H7N9), A(H9N1), and A(H9N2) viruses isolated in Japan (4–6,8) (Table 1). A/chicken/Japan/AQ-HE28-28/2016(H9N2) was detected in illegally brought raw poultry meat (4), and other viruses were detected during surveillance studies for avian influenza A viruses in poultry and wild birds. The antiviral susceptibilities of these viruses were determined through a combination of genotypic methods that detect amino acid substitutions associated with antiviral resistance and phenotypic methods that evaluate phenotypic viral susceptibility to drugs.

The WHO Global Influenza Surveillance and Response System Expert Working Group for Surveillance of Antiviral Susceptibility (WHO-AVWG) published summary tables of amino acid substitutions in the NA and polymerase acidic (PA) subunit proteins that are associated with reduced susceptibility to NA inhibitors and baloxavir, respectively (https://www.who.int/teams/global-influenza-programme/laboratory-network/quality-assurance/antiviral-susceptibility-influenza). No mutant viruses exhibiting reduced susceptibility to favipiravir have emerged in patients infected with influenza after favipiravir treatment, but in vitro studies have shown that a K229R substitution in the polymerase basic subunit 1 protein confers reduced susceptibility to favipiravir (9). Deep sequencing analysis of representative avian influenza A(H5N1), A(H5N2), A(H7N7), A(H7N9), A(H9N1), and A(H9N2) viruses revealed that none of them possessed amino acid substitutions associated with resistance to NA or RNA polymerase inhibitors. The M2 S31N substitution was detected in A/chicken/Ibaraki/1/2005(H5N2) and A/chicken/Japan/AQ-HE28-28/2016(H9N2), demonstrating that these viruses are resistant to M2 inhibitors.

The susceptibilities of avian influenza A(H5N1), A(H5N2), A(H7N7), A(H7N9), A(H9N1), and A(H9N2) viruses to NA and RNA polymerase inhibitors were also examined using a fluorescence-based NA inhibition assay and a focus reduction assay, respectively, as previously described (10) (Table 1). The results were expressed as 50% inhibitory concentration (IC_{50}) values. Human influenza A(H1N1)pdm09 viruses isolated in Japan served as reference viruses: a wild-type virus, an oseltamivir and peramivir cross-resistant virus possessing an H275Y substitution in NA, and a baloxavir-resistant virus possessing an I38T substitution in PA (11). The NA H275Y mutant A(H1N1)pdm09 virus exhibited 990- and 210-fold higher IC_{50} values for oseltamivir and peramivir, respectively, than the wild-type virus. The IC_{50} value of the PA I38T mutant A(H1N1)pdm09 virus against baloxavir was 26-fold higher than that against the wild-type virus. All avian influenza A viruses tested had comparable IC_{50} values to those of previously reported NA inhibitor-susceptible viruses (6,7,12,13). Furthermore, no significant differences in the IC_{50} values for NA and RNA polymerase inhibitors were found between the avian influenza A viruses and the wild-type virus. These results indicate that these representative avian influenza A viruses are susceptible to the NA and RNA polymerase inhibitors approved in Japan.

In Japan, highly pathogenic avian influenza A(H5N8) viruses were detected in poultry (14) and wild birds, and an A(H9N2) virus was detected in raw poultry meat; therefore, we determined the antiviral susceptibilities of these avian influenza A viruses (Table 1). A/chicken/Japan/AQ-HE31-26/2020(H9N2) possessed the M2 S31N substitution, demonstrating M2 inhibitor resistance. The IC_{50} values of these viruses for NA and RNA polymerase inhibitors were similar to those of the representative avian influenza A viruses. These results indicate that avian influenza A viruses recently isolated in Japan are also susceptible to the NA and RNA polymerase inhibitors approved in Japan.

Since global surveillance of antiviral susceptibility is essential for public health planning and for making clinical recommendations for antiviral use, the WHO-AVWG initiated a global analysis of circulating human influenza A and B viruses in the 2012–13 season (15). Monitoring the antiviral susceptibilities of both human and avian influenza viruses is important for minimizing public health risks. However, information regarding the antiviral susceptibilities of avian influenza viruses circulating in Japan is limited. In this study, we found that avian influenza A(H5N1), A(H5N2), A(H5N8), A(H7N7), A(H7N9), A(H9N1), and A(H9N2) viruses isolated in Japan show susceptibility to NA and RNA polymerase inhibitors approved for use in Japan. Furthermore, no amino acid substitutions associated with resistance to these inhibitors were found among the 591 A(H5), 64 A(H7), and 46 A(H9) viruses isolated in Japan that were deposited in the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database (https://www.gisaid.org) (Table 2). Therefore, these drugs could be a treatment option for humans infected by these viruses.

Human-to-human transmission of avian influenza A viruses has rarely been reported; however, human infections continue to occur. Therefore, monitoring the antiviral susceptibilities of circulating avian influenza A viruses should continue for influenza pandemic preparedness.

Table 1. Antiviral susceptibilities of avian influenza A viruses isolated in Japan

Subtype	Isolate name	GISAID isolate ID	Neuraminidase inhibitor[1)]				Polymerase inhibitor[2)]	
			IC_{50} (nM)				IC_{50} (nM)	IC_{50} (μM)
			Oseltamivir	Peramivir	Zanamivir	Laninamivir	Baloxavir	Favipiravir
Representative avian influenza viruses[3)]								
A(H5N1)	A/duck/Hyogo/36/2001	EPI_ISL_3697081	2.22	0.08	0.42	0.26	0.41	1.72
A(H5N2)	A/chicken/Ibaraki/1/2005	EPI_ISL_360	0.13	0.22	1.20	1.31	0.53	0.79
A(H7N7)	A/duck/Fukui/2/2004	EPI_ISL_3707554	0.34	0.23	1.85	2.88	0.36	0.92
A(H7N9)	A/duck/Gunma/466/2011	EPI_ISL_4105027	0.40	0.12	0.77	1.12	0.64	1.44
A(H9N1)	A/duck/Fukui/3/2005	EPI_ISL_3707749	2.05	0.10	0.20	0.21	0.43	1.62
A(H9N2)	A/chicken/Japan/AQ-HE28-28/2016	EPI_ISL_280895	0.09	0.11	0.71	1.24	0.73	2.05
Recently isolated avian influenza viruses								
A(H5N8)	A/chicken/Kagawa/11C/2020 (Clade 2.3.4.4b)	EPI_ISL_681286	0.44	0.06	0.15	0.13	0.26	0.59
A(H5N8)	A/chicken/Miyazaki/E3T/2020 (Clade 2.3.4.4b)	EPI_ISL_4105028	0.53	0.04	0.16	0.10	0.32	0.59
A(H5N8)	A/swan/Niigata/151118/2020 (Clade 2.3.4.4b)	EPI_ISL_4105052	0.31	0.05	0.33	0.33	0.25	0.57
A(H9N2)	A/chicken/Japan/AQ-HE31-26/2020	EPI_ISL_700743	0.11	0.10	0.72	1.15	0.39	1.00
Reference human influenza viruses								
A(H1N1)pdm09	A/KANAGAWA/AC1926/2019 (NA H275Y mutant)[4)]	EPI_ISL_408551	236.53	19.13	0.24	0.86	2.22	1.27
	A/KANAGAWA/IC1890/2019 (PA I38T mutant)[5)]	EPI_ISL_345217	0.29	0.07	0.33	0.30	60.18	0.89
	A/KANAGAWA/ZC1931/2019 (Wild-type)	EPI_ISL_403549	0.24	0.09	0.24	0.19	2.35	1.60
	Mean IC_{50} values[6)] (Wild-type)		0.36 ± 0.16	0.09 ± 0.03	0.31 ± 0.12	0.44 ± 0.23	5.90 ± 2.22	1.08 ± 0.42

GISAID, Global Initiative on Sharing All Influenza Data; IC_{50}, 50% inhibitory concentration; NA, neuraminidase; PA, polymerase acidic subunit.

1): Neuraminidase inhibitor susceptibilities were determined by using a fluorescence-based NA inhibition assay. Viruses were mixed with 20-fold serial dilutions of 125 μM oseltamivir, peramivir, zanamivir, or laninamivir and incubated for 20 min at 37°C; MUNANA substrate (4-(methylumbelliferyl)-N-acetylneuraminic acid) (Biosynth Carbosynth, Berkshire, UK) was then added, and the mixture was incubated for 30 min at 37°C. The fluorescence of the solution was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

2): Polymerase inhibitor susceptibilities were determined by using a focus reduction assay. MDCK cells in 96-well plates were incubated for 24 h at 37°C with 1,000 focus-forming units/well of viruses and 10-fold serial dilutions of 2,500 nM baloxavir or 1,000 μM favipiravir. The cells were then immunostained with a mouse monoclonal antibody against influenza A virus nucleoprotein, followed by a horseradish peroxidase-labelled goat anti-mouse immunoglobulin. The infected cells were stained with TrueBlue Peroxidase Substrate (SeraCare Life Sciences, Milford, MA, USA) and the focus numbers were quantified by using an ImmunoSpot S6 Analyzer (Cellular Technology, Cleveland, OH, USA).

3): The representative avian influenza A viruses were selected based on their subtypes.

4): The H275Y amino acid substitution in NA is associated with reduced susceptibility to oseltamivir and peramivir.

5): The I38T amino acid substitution in PA is associated with reduced susceptibility to baloxavir.

6): Mean IC_{50} values of A(H1N1)pdm09 viruses isolated during the 2019–2020 and 2020–2021 influenza seasons in Japan.

Table 2. Breakdown of sequences of avian influenza A viruses isolated in Japan retrieved from the GISAID EpiFlu database[16)]

Subtype		Number of isolates available for:	
HA	NA	NA gene	PA gene
H5	N1	124	92
	N2	49	26
	N3	33	27
	N6	354	355
	N8	30	30
	N9	1	1
H7	N1	4	3
	N2	3	3
	N3	5	4
	N6	8	8
	N7	35	19
	N8	1	1
	N9	8	8
H9	N1	1	1
	N2	43	38
	N3	1	1
	N4	1	0

GISAID, Global Initiative on Sharing All Influenza Data; HA, hemagglutinin; NA, neuraminidase; PA, polymerase acidic subunit.

Appendix Members of the Influenza Virus Surveillance Group of Japan who participated in the Program of Investigation and Preservation for Influenza Viruses by the Ministry of Health, Labour and Welfare, Japan are as follows: Kazuhiko Ogasawara (Aomori Prefectural Public Health and Environment Center), Ikuo Goto (Miyagi Prefectural Institute of Public Health and Environment), Kenichi Komabayashi (Yamagata Prefectural Institute of Public Health), Asumi Saito (Tochigi Prefectural Institute of Public Health and Environmental Science), Hiroyuki Tsukagoshi (Gunma Prefectural Institute of Public Health and Environmental Sciences), Shinichi Shimada (Saitama Institute of Public Health), Tomoko Ogawa (Chiba Prefectural Institute of Public Health), Aya Kondo (Chiba City Institute of Health and Environment), Mami Nagashima (Tokyo Metropolitan Institute of Public Health), Hideaki Shimizu (Kawasaki City Institute of Public Health), Sachiko Nakamura (Ishikawa Prefectural Institute of Public Health and Environmental science), Kazuo Matsumoto (Fukui Prefectural Institute of Public Health and Environmental Science), Michiko Takeuchi (Nagano Environmental Conservation Research Institute), Masahiro Nishioka (Gifu Prefectural Research Institute for Health and Environmental Sciences), Hideki Suzuki (Shizuoka Institute of Environment and Hygiene), Natsuko Shijo (Hamamatsu City Health Environment Research Center), Takuya Yano (Mie Prefecture Health and Environment Research Institute), Miyano Ojima (Shiga Prefectural Institute of Public Health), Naoki Fujimoto (Kyoto Prefectural Institute of Public Health and Environment), Yuta Tsukamoto (Kyoto City Institute of Health and Environmental Sciences), Satoshi Hiroi and Hideyuki Kubo (Osaka Institute of Public Health), Tomohiro Oshibe (Hyogo Prefectural Institute of Public Health Science), Ai Mori (Kobe Institute of Health), Masaki Yoneda (Nara Prefecture Institute of Health), Yuki Matsui (Wakayama Prefectural Research Center of Environment and Public Health), Ayumi Kawamoto (Tottori Prefectural Institute of Public Health and Environmental Science), Tetsuo Mita (Shimane Prefectural Institute of Public Health and Environmental Science), Yukie Shimazu (Hiroshima Prefectural Technology Research Institute), Yumiko Kawakami (Tokushima Prefectural Public Health, Pharmaceutical and Environmental Sciences Center), Yukari Terajima (Kagawa Prefectural Research Institute for Environmental Sciences and Public Health), Hiromi Iwaki (Ehime Prefectural Institute of Public Health and Environmental Science), Mana Takao (Kochi Public Health and Environmental Science Research Institute), Hideaki Yoshitomi (Fukuoka Institute of Health and Environmental Sciences), Chinami Wasano (Fukuoka City Institute of Health and Environment), Youko Tsutsumi (Saga Prefectural Institute of Public Health and Pharmaceutical Research), Yuji Migita (Nagasaki Prefectural Institute for Environmental Research and Public Health), Mami Sato (Kumamoto Prefectural Institute of Public-Health and Environmental Science), and Katsuya Taira (Okinawa Prefectural Institute of Health and Environment).

Acknowledgments We thank Drs. Yoshihiro Sakoda (Hokkaido University), Makoto Ozawa (Kagoshima University), Toshihiro Ito (Tottori University), and Koichi Otsuki (Kyoto Sangyo University) for their cooperation with the Program of Investigation and Preservation for Influenza Viruses by the Ministry of Health, Labour and Welfare, Japan. We also thank Hiromi Sugawara, Aya Sato, and Miki Akimoto for technical assistance, and Susan Watson for scientific editing. This work was supported by a Grant-in-Aid for Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan [grant numbers 10110400 and 21HA2003], and by JSPS KAKENHI Grant number JP18K10036.

Conflict of interest None to declare.

REFERENCES

1. Bender C, Hall H, Huang J, et al. Characterization of the surface proteins of influenza A(H5N1) viruses isolated from humans in 1997–1998. Virology. 1999;254:115-123.
2. Gao R, Cao B, Hu Y, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013;368:1888-1897.
3. World Health Organization (WHO). Antigenic and genetic characteristics of zoonotic influenza A viruses and development of candidate vaccine viruses for pandemic preparedness. Available at <https://cdn.who.int/media/docs/default-source/influenza/who-influenza-recommendations/vcm-northern-hemisphere-recommendation-2021–2022/202103_zoonotic_vaccinevirusupdate.pdf?sfvrsn=97ae1340_13 >. Accessed July 1, 2022.
4. Shibata A, Okamatsu M, Sumiyoshi R, et al. Repeated detection of H7N9 avian influenza viruses in raw poultry meat illegally brought to Japan by international flight passengers. Virology. 2018;524:10-17.
5. Shibata A, Hiono T, Fukuhara H, et al. Isolation and characterization of avian influenza viruses from raw poultry products illegally imported to Japan by international flight passengers. Transbound Emerg Dis. 2018;65:465-475.
6. Watanabe T, Kiso M, Fukuyama S, et al. Characterization of H7N9 influenza A viruses isolated from humans. Nature. 2013;501:551-555.
7. Imai M, Watanabe T, Kiso M, et al. A highly pathogenic avian H7N9 influenza virus isolated from a human is lethal in some ferrets infected via respiratory droplets. Cell Host Microbe. 2017;22:615-626. e8.
8. Okamatsu M, Saito T, Yamamoto Y, et al. Low pathogenicity H5N2 avian influenza outbreak in Japan during the 2005–2006. Vet Microbiol. 2007;124:35-46.
9. Goldhill DH, Te Velthuis AJW, Fletcher RA, et al. The mechanism of resistance to favipiravir in influenza. Proc Natl Acad Sci U S A. 2018;115:11613-11618.
10. Takashita E, Morita H, Ogawa R, et al. Susceptibility of influenza viruses to the novel cap-dependent endonuclease inhibitor baloxavir marboxil. Front Microbiol. 2018;9:3026.
11. Takashita E, Abe T, Morita H, et al. Influenza A(H1N1)pdm09

virus exhibiting reduced susceptibility to baloxavir due to a PA E23K substitution detected from a child without baloxavir treatment. Antiviral Res. 2020;180:104828.
12. Choi WS, Jeong JH, Kwon JJ, et al. Screening for neuraminidase inhibitor resistance markers among avian influenza viruses of the N4, N5, N6, and N8 neuraminidase subtypes. J Virol. 2018;92:e01580-17.
13. Song MS, Marathe BM, Kumar G, et al. Unique determinants of neuraminidase inhibitor resistance among N3, N7, and N9 avian influenza viruses. J Virol. 2015;89:10891-10900.
14. Sakuma S, Uchida Y, Kajita M, et al. First outbreak of an H5N8 highly pathogenic avian influenza virus on a chicken farm in Japan in 2020. Viruses. 2021;13:489.
15. Takashita E, Daniels RS, Fujisaki S, et al. Global update on the susceptibilities of human influenza viruses to neuraminidase inhibitors and the cap-dependent endonuclease inhibitor baloxavir, 2017–2018. Antiviral Res. 2020;175:104718.
16. Global Initiation on Sharing All Influenza Data (GISAID). Epi Ful database. Available at <https://www.gisaid.org>. Accessed July 1, 2022.