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Running title

Antiviral susceptibility of avian influenza virus

Keywords: Avian influenza; antiviral susceptibility; neuraminidase inhibitor; RNA polymerase inhibitor.

Abstract

Circulation of avian influenza A viruses in poultry is a public health concern because these viruses may cause severe disease in humans and have the potential to become more transmissible among humans. Monitoring the susceptibility of these viruses to antivirals is important for influenza pandemic preparedness. However, information about their antiviral susceptibility 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: the M2 inhibitor amantadine; the neuraminidase inhibitors oseltamivir, peramivir, zanamivir, and laninamivir; and the RNA polymerase inhibitors baloxavir and favipiravir. Genotypic methods that detect amino acid substitutions associated with antiviral resistance and phenotypic methods that assess viral susceptibility to drugs revealed that these avian influenza A viruses are susceptible to neuraminidase inhibitors and RNA polymerase inhibitors. These results suggest that the neuraminidase inhibitors and the RNA polymerase inhibitors currently approved in Japan could be a treatment option against influenza A virus infections in humans.

Text

Circulation of avian influenza A viruses in poultry is a public health concern because these viruses may cause severe disease in humans and have the potential to become more transmissible among humans. In 1997, human infections with a highly pathogenic avian influenza A(H5N1) virus were reported during an outbreak in poultry in Hong Kong (1). Since 2003, this virus has spread and has become endemic in poultry in some countries. The A(H5N1) virus outbreaks have resulted in millions of poultry infections. From January 2003 to November 2021, 863 cases of human infections with A(H5N1) virus were reported across 18 countries. Of these 863 cases, 456 were fatal. In 2013, human infections with an avian influenza A(H7N9) virus were reported in China (2). Since then, this virus has spread in poultry across the country. A total of 1,568 human infections with A(H7N9) virus including 616 fatal cases have been reported since early 2013. Other avian influenza A viruses have been detected sporadically in humans; the most frequently subtypes that have caused human infections are A(H5), A(H7), and A(H9) viruses.

The World Health Organization (WHO) has been assessing the public health risks from avian influenza A(H5), A(H7), and A(H9) viruses and coordinated the development of

candidate vaccine viruses for these subtypes (3). In Japan, avian A(H5) and A(H7) virus outbreaks in poultry have been reported (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 efforts. Furthermore, avian A(H5N1), A(H5N6), A(H7N9), and A(H9N2) viruses were detected in raw poultry meat illegally brought by international flight passengers at the quarantine (4, 5). However, no human cases have been detected in Japan.

For influenza treatment or prophylaxis, three classes of antivirals are approved in Japan: an 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, the WHO does not recommend the use of the M2 inhibitor for treatment of these virus 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 in China from humans (6, 7). However, information about 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 antivirals approved for use there.

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 raw poultry meat illegally brought by international flight passengers (4) and the 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 viral susceptibility to drugs.

The WHO Global Influenza Surveillance and Response System (GISRS) Expert Working Group for Surveillance of Antiviral Susceptibility (WHO-AVWG) published summary tables of amino acid substitutions in the NA and the polymerase acidic subunit (PA) proteins that are associated with reduced susceptibility to the 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 (PB1) protein confers reduced susceptibility to favipiravir (9). Deep sequencing analysis of the representative avian influenza A(H5N1), A(H5N2), A(H7N7), A(H7N9), A(H9N1), and A(H9N2) viruses isolated in Japan revealed that none of them possessed amino acid substitutions associated with resistance to the NA inhibitors or the 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 the M2 inhibitor.

The susceptibilities of the representative avian influenza A(H5N1), A(H5N2), A(H7N7), A(H7N9), A(H9N1), and A(H9N2) viruses isolated in Japan to the NA inhibitors and the RNA polymerase inhibitors were also examined by using a fluorescence-based NA inhibition assay and a focus reduction assay, respectively, as previously described (10) (Table 1). The results are 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 to oseltamivir and peramivir, respectively, than those of the wild-type virus. The IC_{50} value of the PA I38T mutant A(H1N1)pdm09 virus to baloxavir was 26-fold higher than that of the wild-type virus. All avian influenza A viruses tested showed comparable IC_{50} values to the NA inhibitor-susceptible viruses previously reported (6, 7, 12, 13). Furthermore, no significant differences in the IC_{50} values for the 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 inhibitors and the RNA polymerase inhibitors approved in Japan.

Recently 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 illegally brought by international flight passenger; we, therefore, 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 its M2 inhibitor-resistance. The IC_{50} values of these viruses to the 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 inhibitors and the RNA polymerase inhibitors approved in Japan.

Since global surveillance of antiviral susceptibility is essential for public health planning purposes 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 susceptibility of both human and avian influenza viruses is important to minimize public health risk. However, information about the antiviral susceptibilities of avian influenza viruses circulating in Japan has been 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 the NA inhibitors and the RNA polymerase inhibitors approved for use in Japan. Furthermore, no amino acid substitutions associated with resistance to these inhibitors were found among 589 A(H5), 64 A(H7), and 46 A(H9) viruses isolated in Japan that had been deposited in the GISAID EpiFlu database (https://www.gisaid.org) (Table 2). Therefore, these drugs could be a treatment option for humans infected with these viruses.

Human-to-human transmission of avian influenza A viruses has been reported rarely but human infections will continue to occur. Monitoring the antiviral susceptibility of circulating avian influenza A viruses should, therefore, continue to be conducted for

influenza pandemic preparedness.

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 Sciences), 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

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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;254:115-23.

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;368:1888-97.

3. World Health Organization. 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 >.

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;524:10-7.

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;65:465-75.

6. Watanabe T, Kiso M, Fukuyama S, et al. Characterization of H7N9 influenza A viruses isolated from humans. Nature. 2013;501:551-5.

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;22:615-26 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;115:11613-8.

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;89:10891-900.

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.

Tables

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	<u>236.53</u>	<u>19.13</u>	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	<u>60.18</u>	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.

Tables

Table 2. Breakdown of sequences of avian influenza A viruses isolated in Japan retrieved from the GISAID EpiFlu database[1)]

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.

[1)] Accessed December 7, 2021.