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ORIGINAL ARTICLE

Pathogenicity of two novel human-origin H7N9 highly pathogenic avian influenza viruses in chickens and ducks

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Abstract

Human infection by low-pathogenic avian influenza viruses of the H7N9 subtype was first reported in March 2013 in China. Subsequently, these viruses caused five outbreaks through September 2017. In the fifth outbreak, H7N9 virus possessing a multiple basic amino acid insertion in the cleavage site of hemagglutinin emerged and caused 4% of all human infections in that period. To date, H7N9 highly pathogenic avian influenza viruses (HPAIVs) have been isolated from poultry, mostly chickens, as well as the environment. To evaluate the relative infectivity of these viruses in poultry, chickens and ducks were subjected to experimental infection with two H7N9 HPAIVs isolated from humans, namely A/Guangdong/17SF003/2016 and A/Taiwan/1/2017. When chickens were inoculated with the HPAIVs at a dose of 10^6 50% egg infectious dose (EID_{50}), all chickens died within 2–5 days after inoculation, and the viruses replicated in most of the internal organs examined. The 50% lethal doses of A/Guangdong/17SF003/2016 and A/Taiwan/1/2017 in chickens were calculated as $10^{3.3}$ and $10^{4.7}$ EID_{50}, respectively. Conversely, none of the ducks inoculated with either virus displayed any clinical signs, and less-efficient virus replication and less shedding were observed in ducks compared to chickens. These findings indicate that chickens, but not ducks, are highly permissive hosts for emerging H7N9 HPAIVs.

Introduction

The first case of human infection by influenza subtype H7N9, a novel low-pathogenic avian influenza virus (LPAIV), was reported in March 2013 in Shanghai and Anhui, China. The virus caused five epidemics through September 2017. Subsequently, three laboratory-confirmed cases, including one death, were reported by the World Health Organization (WHO) [1]. The fifth epidemic, which started on October 1, 2016, was the largest, comprising 766 laboratory-confirmed cases, including 248 lethal cases [2]. As of May 28, 2018, a total of 1567 laboratory-confirmed cases have been reported, including 615 deaths [1]. The human cases included one patient from Malaysia and two patients from Canada, all of whom had recently visited China [3, 4].

The first H7N9 epidemic occurred mainly in the Yangtze River Delta (YRD) region, which includes Zhejiang, Jiangsu, and Anhui provinces and Shanghai municipality (111/135 cases: 82%). In the second epidemic, the second-highest number of cases after the YRD region (144/320: 45%) was recorded in the Pearl River Delta (PRD) region (118/320 cases: 37%), which includes Guangdong province and the Hong Kong and Macao special administrative

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regions. Before the fourth epidemic, most human cases of H7N9 infection were primarily reported in these regions (623 out of 798 cases, 78%). However, in the fifth epidemic, the infection was widely distributed in other regions of China (378 out of 766 cases; 49%). In addition, during this outbreak, infection by emerging highly pathogenic avian influenza viruses (HPAIVs) of the H7N9 subtype that had acquired a multiple basic amino acid insertion in the cleavage site of hemagglutinin (HA) was observed, specifically 32 cases in Guangxi, Guangdong, Hunan, Shaanxi, Hebei, Henan, Fujian, and Yunnan provinces, as well as in Chinese Taipei [5, 6]. Through phylogenetic analysis using the HA gene, the emerging H7N9 viruses were mainly divided into two main lineages: the YRD lineage and the PRD lineage [7]. The HPAIVs were found to have originated from H7N9 LPAIVs belonging to the YRD lineage [8]. In this same period, H7N9 HPAIVs belonging to the same lineage were also isolated from chickens and environmental samples in live-bird markets (LBMs) and farms [9–12].

A/Anhui/1/2013, the H7N9 virus isolated in the first human case, causes fatal infection in mice and asymptomatic infection in chickens [13–16]. Recently emerging HPAIVs of the H7N9 subtype cause high mortality and exhibit efficient propagation in chickens; contrarily, they differentially replicate in the internal organs of Pekin ducks, which exhibit variable virus shedding rates of 0%–60% without causing symptoms, depending on the strain [11, 17]. In the poultry outbreak caused by H7N9 HPAIVs, approximately 128,000 birds died and 942,000 birds were destroyed as of September 16, 2018 [9]. According to FAO statistics on September 5, 2018, 41 of 43 locations where HPAIVs of the H7N9 subtype were isolated were LBMs and farms [18]. Of the 49 viruses isolated from the birds and the environment, most avian isolates were obtained from chickens, and only two isolates were obtained from ducks [18]. In this study, experimental infection of chickens and ducks using H7N9 HPAIVs isolated from humans was performed, and the preferential infectivity of the H7N9 HPAIVs among poultry was evaluated.

Materials and methods

Viruses

A/Anhui/1/2013 (Anhui2013) [19] and A/Guangdong/17SF003/2016 (Guangdong2016) [20] were provided by the Chinese Center for Disease Control and Prevention (CCDC) through the National Institute of Infectious Diseases (NIH) in Japan. A/Taiwan/1/2017 (Taiwan2017) was isolated at the Centers for Disease Control in Chinese Taipei as described previously [21]. The original Guangdong2016 and Taiwan2017 strains were passaged twice and once, respectively, at the respective institutes. Subsequently, they were passaged once at NIH and once at the National Institute of Animal Health, NARO, Japan, in embryonated eggs. Guangdong2016 and Taiwan2017 were subjected to genetic analysis of viral RNA and animal experiments. Anhui2013 was passaged four times in embryonated eggs as described previously [16].

Genetic analysis

Viral RNA was extracted from the allantoic fluid of embryonated eggs by using an RNeasy Mini Kit (QIAGEN). The cDNA library was synthesized using a NEBNext Ultra RNA Library Prep Kit (NEB). Viral genome sequencing was performed using a Miseq sequencer and a Miseq Reagent Kit v2 (Illumina). The consensus sequences were generated using FLUGAS software (version 0.9.0, World Fusion, Tokyo, Japan). The DNA sequences of Guangdong2016 and Taiwan2017 were deposited in Global Initiative on Sharing Avian Influenza Data (GISAID; http://platform.gisaid.org/) (Isolate ID: EPI_ISL_280902, EPI_ISL_280894). For comparison of the deduced amino acid sequences, 24 HPAIVs of H7N9 subtype isolated from chickens, which were full-length sequenced, A/Chicken/Huizhou/HZ-3/2016 (EPI_ISL_248796), A/Chicken/Guangzhou/HD621/2017 (EPI_ISL_248816), A/Chicken/Huizhou/HZX/2017 (EPI_ISL_248886), A/Chicken/Zhongshan/ZS/2017 (EPI_ISL_249113), A/Chicken/Huizhou/HZ04/2016 (EPI_ISL_249114), A/chicken/Guangdong/01.08 SZBJ0011-O/2017 (EPI_ISL_259758), A/chicken/Shandong/05.05 DZ056/2017 (EPI_ISL_276783), A/Dressed_chicken/Guangdong/GZ631/2017 (EPI_ISL_273950), A/chicken/Heinan/ZZ01/2017 (EPI_ISL_274206), A/chicken/Fujian/06.06 NP0001/2017 (EPI_ISL_276785), A/chicken/Guangdong/J1/2017 (EPI_ISL_280466), A/chicken/Guangdong/J2/2017 (EPI_ISL_280467), A/chicken/Guangdong/SD008/2017 (EPI_ISL_283486), A/chicken/Guangdong/SD010/2017 (EPI_ISL_283487), A/chicken/Guangdong/SD027/2017 (EPI_ISL_283495), A/chicken/Guangdong/SD028/2017 (EPI_ISL_283496), A/chicken/Guangdong/SD031/2017 (EPI_ISL_283498), A/chicken/Guangdong/SD032/2017 (EPI_ISL_283499), A/chicken/Guangdong/SD034/2017 (EPI_ISL_283500), A/chicken/Guangdong/30/2017 (accession no.: MF184011-MF184018), A/chicken/Guangdong/GD20/2017 (KY751060-KY751290), A/chicken/Guangdong/Q26/2017 (MF280182- MF280203), A/chicken/Guangdong/Q39/2017 (MF280183-MF280204), and A/chicken/Heilongjiang/BQC01/2017 (MG298777-MG298784) from the GISAID and the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) (accessed 16 September 2018) were used in this study.

Animal experiments

Four-week-old specific-pathogen-free white leghorn chickens and 4-week-old Cherry Valley strain domestic ducks were purchased from Nissei Bio Co., Ltd. (Tokyo, Japan) and Hamada Co. Ltd (Saitama, Japan), respectively. All animal experiments were conducted in biosafety level 3 facilities at the National Institute of Animal Health, Japan, and approved by the committee for the institute's ethics of animal experiments. For survival analysis, each group of four chickens was inoculated intranasally with virus doses of 10^2, 10^4, and 10^6 50% egg infectious dose (EID_{50})/100 µL, and four ducks were inoculated with a dose of 10^6 EID_{50}/100 µL. The chickens and ducks were observed for 14 days postinoculation (dpi), and tracheal and cloacal swabs were collected on 1, 2, 3, 5, 7, 10, and 14 dpi or at the time of death for virus titration. Swabs were dipped in MEM containing 0.5% BSA, 25 µg of Fungizone per mL, 1000 units of penicillin per mL, 1000 µg of streptomycin per mL, 0.01 M HEPES, and 8.8 mg of $NaHCO_3$ per mL and then removed from the medium. The medium was then stored at −80°C until titration. To assess viral dissemination in chicken and duck bodies, nine chickens and nine ducks were inoculated with a virus dose of 10^6 EID_{50}/100 µL. Three chickens each were euthanized at 2, 3, and 4 dpi, and three ducks each were euthanized at 2, 4, and 6 dpi. Tracheal, cloacal, and conjunctival swab specimens were taken before euthanasia, and 12 tissues and organs (blood, pancreas, spleen, muscle, liver, trachea, lungs, kidneys, heart, brain, duodenum, and rectum) were excised. Homogenates of the organs were prepared in MEM containing 0.5% BSA, 25 µg of Fungizone per mL, 1000 units of penicillin per mL, 1000 µg of streptomycin per mL, 0.01 M HEPES, and 8.8 mg of $NaHCO_3$ per mL as described previously [16] and stored at −80°C until titration. The viral titers in the swab specimens and organ homogenates were calculated as EID_{50} using the Reed and Muench method [22]. Blood samples in surviving chickens or ducks were taken at the end of the observation period for antibody detection. Antibodies against influenza type A virus were detected via ELISA and the HA inhibition (HI) test.

Antibody detection

To detect antibodies against influenza type A viruses, ELISA was performed using an influenza A virus antibody test kit (IDEXX Laboratories, Inc.). Antibodies against the HA proteins of the viruses were detected using an HI test according to the WHO Manual on Animal Influenza Diagnosis and Surveillance [23]. The detection limit of the HI test was defined as a titer of <4, as the lowest serum dilution was 1:4.

Statistical analysis

Survival analysis was performed using the Kaplan–Meier method, and survival differences were analyzed using the log-rank test. The differences of viral titers in the swab specimens between Guangdong2016 and Taiwan2017 were analyzed by the Mann–Whitney U-test.

Results

Pathogenicity of H7N9 HPAIVs in chickens

The lethality and replication of the human-derived H7N9 HPAIVs in chickens were examined via intranasal inoculation with Guangdong2016 or Taiwan2017. As shown in Fig. 1, all chickens inoculated with 10^6 EID_{50} of Guangdong2016 and Taiwan2017 died by 5 dpi, whereas 75% and 25%, respectively, of chickens inoculated with 10^4 EID_{50} of these two viruses died by 14 dpi. None of the chickens inoculated with 10^2 EID_{50} of either virus died or exhibited clinical symptoms. Chickens inoculated with Taiwan2017 displayed reduced activity 1–3 days before death, and depression and cyanosis of the comb and/or legs were observed on the day of or 1–2 days before death. Facial edema was also observed 1–2 days before death in all four chickens that were infected with a dose of 10^6 EID_{50}. Chickens infected with Guangdong2016 exhibited similar clinical symptoms as those infected with Taiwan2017, but one chicken each in the 10^4 and 10^6 EID_{50} dose groups displayed no clinical symptoms one day before death. Based on the survival rates, the 50% chicken lethal doses (CLD_{50}) of Guangdong2016 and Taiwan2017 were calculated as $10^{3.3}$ and $10^{4.7}$ EID_{50}, respectively. At a dose of 10^6 EID_{50}, the mean durations between

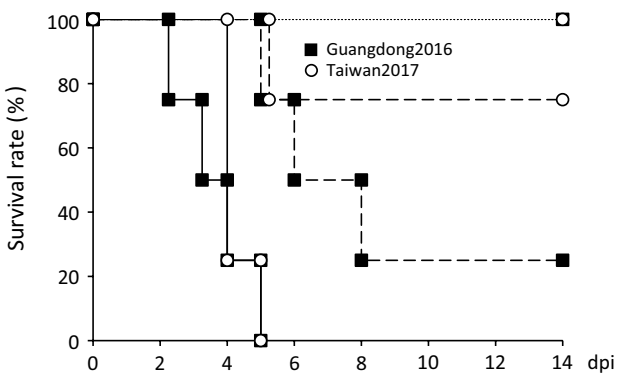

Fig. 1 Survival rates of chickens inoculated intranasally with 10^2 (dotted line), 10^4 (dashed line), or 10^6 (solid line) 50% egg infective dose of highly pathogenic avian influenza viruses of the H7N9 subtype (four chickens per group). The Guangdong2016 and Taiwan2017 viruses are represented by closed squares and open circles, respectively. dpi, days postinoculation

inoculation and death in the Guangdong2016 and Taiwan2017 groups were 3.6 and 4.3 days, respectively, which was not a statistically significant difference. At 1 dpi, shedding of Guangdong2016 was detected in cloacal swabs from three of four chickens (1.60 ± 0.57 $\log_{10}$ EID_{50}/mL), whereas that of Taiwan2017 was not observed. Regarding viral shedding, the mean highest titer prior to death in the cloaca of chickens infected with 10^6 EID_{50} of Guangdong2016 (4.51 ± 0.55 $\log_{10}$ EID_{50}/mL) was significantly higher than that for the same dose of Taiwan2017 (3.40 ± 0.42 $\log_{10}$ EID_{50}/mL) (Table 1). At the time of death, there was no significant difference in the average viral titers between the Guangdong2016 and Taiwan2017 groups. Among the chickens that survived for 14 dpi after inoculation with a virus dose of 10^2 or 10^4 EID_{50}, two of five inoculated with Guangdong2016 and three of seven inoculated with Taiwan2017 shed small amounts of virus (0.32 $\log_{10}$ EID_{50}/mL) at 1 or 2 dpi. However, according to ELISA, these animals did not exhibit seroconversion (Table 1). The 50% chicken infectious doses of Guangdong2016 and Taiwan2017 were calculated as $10^{2.7}$ and $10^{3.0}$ EID_{50}, respectively. As shown in Table 2, when chickens were infected with 10^6 EID_{50} of Guangdong2016 or Taiwan2017, the viruses were detected at average titers of 2.47–5.61 and 2.32–6.38 $\log_{10}$ EID_{50}/g tissue or mL, respectively, using tissue and swab specimens at 2, 3, and 4 dpi, excluding one chicken inoculated with Taiwan2017. The data indicated that human-derived H7N9 HPAIVs are highly pathogenic to chickens.

Pathogenicity of H7N9 HPAIVs in ducks

Intranasal inoculation with 10^6 EID_{50} of the viruses was also performed in domestic ducks. In this experiment, Anhui2013, an ancestral strain of the HPAIVs, was also included to evaluate viral pathogenicity in ducks. As shown in Table 3, all ducks survived for 14 days after challenge with any of the strains without any clinical symptoms. Two of four ducks inoculated with Guangdong2016 shed the virus. One duck shed the virus from 1 to 5 dpi in the trachea and at 2 dpi in the cloaca, whereas the second duck shed the virus in the cloaca at 2 dpi. The highest titer of 4.02 $\log_{10}$ EID_{50}/mL was observed in the trachea in the first duck at 3 dpi. Seroconversion was detected in the first duck via ELISA, but not by the HI test with homologous antigen. The latter duck had not seroconverted according to either assay; however, two other ducks that did not shed virus had seroconverted according to the ELISA readout. Only one duck that was inoculated with Taiwan2017 shed the virus at 3 and 5 dpi in the trachea and at 3 dpi in the cloaca, and this animal had seroconverted. No other animal inoculated with this virus exhibited seroconversion. The highest titer in the trachea of 3.02 $\log_{10}$ EID_{50}/mL was observed at 3 dpi. Among the ducks inoculated with Anhui2013, only one duck shed virus in the cloaca at 7 dpi, and none of the ducks seroconverted. As shown in Table 2, no virus was detected in any tissues, excluding the cloacal swab of a duck inoculated with Taiwan2017 that was euthanized at 6 dpi. These results clearly indicated that human-derived H7N9 viruses were non-lethal for ducks, and their replication was extremely low compared with that in chickens.

Comparison of amino acid sequences between the H7N9 HPAIVs

To evaluate differences in the amino acid sequences of the viral proteins of the two human isolates and chicken isolates of H7N9 HPAIV and to identify amino acid changes in viral proteins that occurred through egg passage from the original strains [20, 21], the deduced amino acid sequences of the two human isolates were compared with a consensus sequence derived from 24 chicken H7N9 HPAIV isolates obtained from the GISAID and the NCBI database. As shown in Table 4, over half of the consensus amino acid residues (27/43) were conserved in Guangdong2016, whereas only 15 residues were conserved in Taiwan2017. Taiwan2017 has a potential glycosylation site at positions 128–130 in HA1, unlike Guangdong2016 and the chicken isolates. In addition, the C-terminal region of NS1 of Guangdong2016 and the chicken isolates was truncated by 20 amino acids compared with that of Taiwan2017. After several viral passages in eggs, six amino acid substitutions had occurred in PB2, HA, neuraminidase (NA), and NS2. The substituted amino acids in HA and NS2 of Taiwan2017 and PB2 of Guangdong2016 were predominant at the second and fourth passages, whereas those in HA and NA of Guangdong2016 were already predominant at the third passage. Glycine at position 129 (H3 numbering) of HA of Guangdong2016, which was near the receptor-binding pocket, was substituted by glutamic acid. The lysine residue at position 292 (N2 numbering) of NA of Guangdong2016, which influences sensitivity to NA inhibitors, was substituted by an arginine residue.

Discussion

The high pathogenicity of human H7N9 HPAIVs in chickens was similar to that of H7N9 HPAIVs isolated from chickens by another group, which belonged to the same phylogenetic tree cluster of the HA gene as Taiwan2017 and Guangdong2016. The pathogenicity of these H7N9 HPAIVs in chickens was apparently increased over that of Anhui2013, their ancestral LPAIV of human origin [16]. Meanwhile, the differences in pathogenicity and viral shedding between Taiwan2017 and Guangdong2016 might be attributable to amino acid substitutions relative to the consensus sequences

Table 1 Viral titers in trachea and cloacal swabs collected from live or dead chickens inoculated with Guangdong2016 or Taiwan2017 during the observation period[a]

Virus	Inoculation dose (EID_{50}/head)		Log_{10} EID_{50}/ml at each dpi													Average maximum viral titer in live chickens	Average viral titer at the time of death
			1	2		3		4	5		6	7	8	10	14		
			live[b]	live	dead[c]	live	dead	dead	live	dead	dead	live	dead	live	live		
Guangdong2016	10^2	Trachea	< (0/4)	< (0/4)	–	< (0/4)	–	–	< (0/4)	–	–	< (0/4)	–	< (0/4)	< (0/4)	< (0/4)	–
		Cloaca	< (0/4)	0.32 (1/4)	–	< (0/4)	–	–	< (0/4)	–	–	< (0/4)	–	< (0/4)	< (0/4)	0.32 (1/4)	–
	10^4	Trachea	< (0/4)	1.32 ± 1.41 (2/4)	–	3.14 ± 1.33 (2/4)	–	–	5.87 (1/3)	7.20 (1/4)	6.87 (1/3)	3.20 (1/2)	6.02 (1/2)	< (1/1)	< (1/1)	3.37 ± 2.31 (4/4)	6.69 ± 0.61 (3/4)
		Cloaca	< (0/4)	0.43 ± 0.15 (2/4)	–	3.73 ± 0.49 (2/4)	–	–	3.20 (1/3)	3.32 (1/4)	2.53 (1/3)	4.20 (1/2)	3.87 (1/2)	< (1/1)	< (1/1)	2.99 ± 1.82 (4/4)	3.24 ± 0.67 (3/4)
	10^6	Trachea	1.70 ± 0.66 (3/4)	3.18 ± 0.84 (4/4)	4.32 (1/4)	4.13 ± 1.09 (3/3)	5.32 (1/3)	7.20 (1/2)	N.A	6.32 (1/1)	N.A	N.A	N.A	N.A	N.A	4.18 ± 0.89 (4/4)	5.79 ± 1.24 (4/4)
		Cloaca	1.60 ± 0.57 (3/4)	4.48 ± 0.57 (4/4)	5.32 (1/4)	3.51 ± 0.71 (3/3)	3.02 (1/3)	5.32 (1/2)	N.A	4.32 (1/1)	N.A	N.A	N.A	N.A	N.A	4.51 ± 0.55 (4/4)*	4.50 ± 1.09 (4/4)
Taiwan2017	10^2	Trachea	< (0/4)	0.32 (1/4)	–	< (4/4)	–	–	< (4/4)	–	–	< (4/4)	–	< (4/4)	< (4/4)	0.32 (1/4)	–
		Cloaca	0.32 (1/4)	< (0/4)	–	< (4/4)	–	–	< (4/4)	–	–	< (4/4)	–	< (4/4)	< (4/4)	0.32 (1/4)	–
	10^4	Trachea	< (0/4)	0.45 ± 0.18 (2/4)	–	1.32 (1/4)	–	–	5.87 (1/4)	5.87 (1/4)	–	< (3/3)	–	< (3/3)	< (3/3)	3.09 ± 3.92 (2/4)	5.87 (1/4)
		Cloaca	< (0/4)	< (0/4)	–	3.20 (1/4)	–	–	< (4/4)	< (1/4)	–	< (3/3)	–	< (3/3)	< (3/3)	3.2 (1/4)	< (1/4)
	10^6	Trachea	1.12 ± 0.43 (4/4)	2.23 ± 1.22 (4/4)	–	3.24 ± 0.84 (4/4)	–	5.87 ± 0.43 (3/4)	N.A	5.02 (1/1)	N.A	N.A	N.A	N.A	N.A	3.24 ± 0.84 (4/4)	5.65 ± 0.55 (4/4)
		Cloaca	< (0/4)	2.84 ± 0.50 (3/4)	–	3.15 ± 0.69 (4/4)	–	3.37 ± 2.10 (3/4)	N.A	5.20 (1/1)	N.A	N.A	N.A	N.A	N.A	3.40 ± 0.42 (4/4)*	3.83 ± 1.94 (4/4)

[a]Swabs were collected at 1, 2, 3, 5, 7, 10, and 14 dpi from live birds and on the day they died

[b]Numbers in parentheses, number of chickens that were positive in virus isolation/number of live chickens at the time of sampling; <, no virus was detected from the specimens collected during the observation period (detection limit < 0.20 log_{10} EID_{50}/mL); N.A, Not applicable as all chickens died in the group

[c]Numbers in parentheses, number of dead chickens/number of live chickens before the time of sampling; –, no chicken died in the group; <, no virus was detected from the specimens collected during the observation period (detection limit < 0.20 log_{10} EID_{50}/mL)

*, $P < 0.05$ between Guangdong2016 and Taiwan2017

Table 2 Dissemination of the viruses in chickens and ducks inoculated with Guangdong2016, Taiwan2017, or Anhui2013[a]

Virus	Host[b]	Animal number	Collection days (dpi)	Tracheal swab	Cloacal swab	Conjunctiva swab	Blood	Pancreas	Spleen	Muscle	Liver	Trachea	Lungs	Kidneys	Heart	Brain	Duodenum	Rectum
				(Log_{10} EID_{50}/ml)[d]			(Log_{10} EID_{50}/g tissue)											
Guangdong2016	Chicken[c]	1	2	3.70	4.32	3.02	5.53	7.20	6.20	5.07	5.07	6.20	5.87	5.87	5.53	5.32	6.53	4.87
		2		2.02	4.53	<	4.20	<	5.32	3.20	4.32	4.20	5.53	5.32	4.32	4.20	4.20	4.07
		3		2.32	4.53	0.32	3.38	4.20	4.87	3.07	4.87	4.07	5.53	5.87	4.87	4.20	4.20	3.53
		4	3	4.20	3.53	3.32	5.38	2.70	6.53	5.87	5.32	6.87	7.53	6.53	6.02	6.70	6.07	5.87
		5		2.87	2.02	1.32	4.20	7.53	5.20	3.32	5.02	5.53	6.32	6.20	5.32	5.87	5.02	4.20
		6		5.07	5.07	2.53	5.53	6.32	5.32	5.20	5.32	5.53	7.70	6.38	5.32	7.20	4.53	4.53
		7	4	5.32	1.20	3.38	3.87	8.32	5.70	4.20	4.32	6.53	6.20	6.20	5.20	7.07	5.07	4.02
	Duck	1	2	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		2		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		3		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		4	4	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		5		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		6		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		7	6	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		8		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		9		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Taiwan2017	Chicken	10	2	1.87	4.32	0.32	2.20	3.20	3.87	2.53	4.38	4.53	5.20	5.07	4.20	3.87	3.32	4.02
		11		2.02	1.07	1.87	2.53	3.32	4.32	3.38	4.20	5.20	5.20	5.02	5.38	5.07	1.20	3.87
		12		1.70	2.53	<	2.07	2.53	4.07	2.87	3.87	5.07	4.70	4.70	5.20	4.02	3.45	3.07
		13	3	5.02	1.87	2.02	3.07	3.53	5.32	3.07	4.53	5.07	6.20	4.87	5.32	5.20	4.32	4.20
		14		3.53	<	3.32	4.20	5.07	5.87	4.32	5.20	6.20	6.20	5.53	5.53	5.32	5.07	5.20
		15		6.07	3.02	4.38	5.87	5.53	6.32	6.38	6.32	6.87	6.20	7.32	6.53	6.70	5.53	5.87
		16	4	4.53	<	3.53	3.32	5.07	4.53	3.87	4.87	6.02	5.32	5.53	5.53	7.32	4.32	4.20
		17		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		18		3.32	3.20	1.87	3.07	3.87	5.20	3.07	3.70	5.20	5.87	5.32	4.53	5.20	3.53	3.07
	Duck	10	2	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		11		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		12		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		13	4	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		14		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		15		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		16	6	<	0.32	<	<	<	<	<	<	<	<	<	<	<	<	<
		17		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		18		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<

Table 2 (continued)

Virus	Host[b]	Animal number	Collection days (dpi)	Tracheal swab	Cloacal swab	Conjunctiva swab	Blood	Pancreas	Spleen	Muscle	Liver	Trachea	Lungs	Kidneys	Heart	Brain	Duodenum	Rectum
				(Log_{10} EID_{50}/ml)[d]			(Log_{10} EID_{50}/g tissue)											
Anhui2013	Duck	19	2	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		20		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		21		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		22	4	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		23		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		24		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		25	6	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		26		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
		27		<	<	<	<	<	<	<	<	<	<	<	<	<	<	<

[a]Nine head/group were intranasally inoculated with 10^6 EID_{50} of the virus

[b]Three chickens and ducks were euthanized at 2, 3, and 4 dpi and 2, 4, and 6 dpi, respectively, and their specimens were collected

[c]Two out of nine chickens inoculated with the virus died before the scheduled euthanasia

[d]<, No virus was detected from the specimens collected (detection limit < 0.20 log_{10} EID_{50}/mL or EID_{50}/g tissue)

of avian H7N9 HPAIVs. Taiwan2017 exhibited a higher degree amino acid substitution, suggesting that Guangdong2016 retained more common features with chicken-origin H7N9 HPAIV. One amino acid substitution that arose in Guangdong2016 after egg passages was Lys292Arg in NA, which has been linked to reduced sensitivity to NA inhibition and less efficient replication in eggs for a human H3N2 isolate [24]. Reversion of this amino acid might increase the replication of Guangdong2016 in chickens. In addition, threonine at position 130 of HA1 in Taiwan2017 generates an N-glycosylation site at position 128. The position is located near the receptor-binding site, and glycosylation at this site might affect the receptor preference of the virus, resulting in less efficient replication in chickens. In line with this hypothesis, deletion of N-oligosaccharides at Asn123 or Asn149 in the HA protein of the H7 subtype has been reported to enhance hemadsorbing activity, suggesting that N-glycosylation in the vicinity of the receptor-binding site can interfere with receptor-binding activity [25]. Meanwhile, it was reported that a 20-amino acid extension of the C-terminus of the NS1 protein enhanced the replication and transmissibility of H9N2 strains in chickens [26]. However, only Taiwan2017 was found to possess this extension. As the pathogenicity of HPAIVs is a multi-gene trait, further investigation is necessary to elucidate the contribution of these substitutions to the pathogenicity of H7N9 HPAIVs in chickens.

Chickens possess both α2,6- and α2,3-linked sialic acids in their upper respiratory and intestinal tracts, whereas ducks mainly have α2,3-linked sialic acids, with α2,6-linked sialic acids being absent from their intestinal tracts [27]. These differences could partially explain the differences in the pathogenicity of the investigated viruses between chickens and ducks. The short length of the NA protein might also contribute to these differences. The H7N9 avian influenza viruses circulating in mainland China, including those used in this study, have a 5-amino-acid deletion in the stalk of the NA protein. An H7N1 LPAIV bearing a short-stalk NA was excreted in low titers from the cloaca of virus-infected Pekin ducks, whereas high titers of virus were shed from the oropharynx of virus-infected chickens compared to a recombinant virus that featured a 22-amino-acid insertion in the short-stalk NA [28]. It has also been reported that a 30-amino-acid deletion in an H11N9 virus hampered its replication in Pekin ducks but not in chickens [29]. The influence of the 5-amino-acid deletion in the NA protein of the H7N9 viruses on replication in ducks and chickens will need to be studied further. Meanwhile, there are some differences in immune-related genes between chickens and ducks, including RIG-I, which predominantly functions as an anti-influenza-virus sensor in ducks [30]. The absence of this gene in chickens has been suggested as a cause of their

Table 3 Viral titers in trachea and cloacal swabs collected from ducks inoculated with 10^6 EID_{50} of Guangdong2016, Taiwan2017, or Anhui2013 during the observation period and antibody detection results[a]

Virus	Animal number		Log_{10} EID_{50}/ml at each dpi[b]							Antibody detection[c]	
			1	2	3	5	7	10	14	ELISA	HI
Guangdong2016	1	Trachea	0.87	3.45	4.02	3.32	<	<	<	+	<
		Cloaca	<	0.32	<	<	<	<	<		
	2	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	0.53	<	<	<	<	<		
	3	Trachea	<	<	<	<	<	<	<	+	<
		Cloaca	<	<	<	<	<	<	<		
	4	Trachea	<	<	<	<	<	<	<	+	<
		Cloaca	<	<	<	<	<	<	<		
Taiwan2017	5	Trachea	<	<	3.02	0.53	<	<	<	+	<
		Cloaca	<	<	0.32	<	<	<	<		
	6	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	<	<	<	<	<	<		
	7	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	<	<	<	<	<	<		
	8	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	<	<	<	<	<	<		
Anhui2013	9	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	<	<	<	0.32	<	<		
	10	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	<	<	<	<	<	<		
	11	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	<	<	<	<	<	<		
	12	Trachea	<	<	<	<	<	<	<	−	<
		Cloaca	<	<	<	<	<	<	<		

[a]Specimens were collected at 1, 2, 3, 5, 7, 10, and 14 days postinoculation (dpi)

[b]<, No virus was detected from the specimens collected (detection limit < 0.20 EID_{50}/mL)

[c]− or +, The serum in surviving ducks at 14 dpi was negative or positive using the influenza A virus antibody test kit (IDEXX Laboratories, Inc.); <, No antibody was detected by HI test (detection limit < 4)

greater susceptibility to influenza virus infection compared to ducks [31].

In line with our findings in ducks, prior research using Pekin ducks and other H7N9 HPAIVs, namely A/chicken/Heyuan/16876/2016, A/Chicken/Huizhou/HZ-3/2016, A/Guangdong/Th005/2017, and A/Guangdong/Th008/2017, produced concordant findings [11]. These viruses, excluding A/Chicken/Huizhou/HZ-3/2016, were shed by Pekin ducks to some extent, whereas no shedding was observed for A/Chicken/Huizhou/HZ-3/2016. The low-pathogenic strain Anhui2013 rarely replicated in Cherry Valley ducks in our study, whereas another group reported that the virus replicated well and was shed at relatively high titers in oropharyngeal swabs from Pekin ducks [32]. Meanwhile, findings similar to ours have also been reported by another group who used Cherry Valley ducks as experimental models of infection [33]. The difference in sensitivity to influenza viruses between these two duck species might be related to differences in age at the time of experimentation, namely 4 weeks for Cherry Valley ducks and 2 weeks for Pekin ducks, as age-related effects have been reported previously for HPAIV infection [34].

To date, H7N9 HPAIVs have never been isolated from wild birds. However, these viruses may have the potential to acquire the ability to infect migratory birds via genetic mutation and genetic reassortment between viruses. Previously, an Asian H5 HPAIV caused an outbreak at a goose farm in Guangdong province in China in 1996 [35]. After a long period, the progeny viruses invaded North America and were rapidly disseminated in 2014–2015 [36, 37]. This rapid dissemination is reportedly associated with long-distance migratory birds [38]. In Japan, data revealed that the isolation rate of HPAIVs from dead wild birds rapidly increased to 9% in 2016–2017, versus a rate of 0%–1% between the start of surveillance in 2008 and 2016 [39]. It has been reported that when mallard ducks were infected by HPAIVs isolated in North America in 2014, they shed more viruses in the oropharynx than ducks infected with 11 of the 12 other

Table 4 Comparative alignment of deduced amino acid sequences among Guangdong2016, Taiwan2017, and HPAIVs of H7N9 subtype isolated from chickens[a]

	Host	PB2								PB1					PB1-F2		
		82	152	482	560	584	627	648	702	372	566	635	642	646	41	42	57
Guangdong2016	Human	**K**	A	R	I	I	E	V	R	M	T	R	N	M	R	C	Y
Taiwan2017		N	T	K	V	V	K	I	K	L	M	K	S	L	H	Y	C
Consensus sequence[b]	Chicken	N/V	A	K	V	V	E	V	K	M	T	K	N	M	H	Y	Y

		PA						PA-X		HA1[c]				HA2[c]	NP	
		66	228	256	465	497	556	66	228	137 (129)	138 (130)	181 (172)	279 (270)	42 (42)	89	399
Guangdong2016	Human	G	N	R	V	R	Q	G	T	**E**	I	K	R	Q	P	Q
Taiwan2017		S	S	K	I	K	H	S	A	G	T	R	G	**R**	H	H
Consensus sequence	Chicken	G	N	R	I	K	Q	G	T	G	I	R	G	Q	P	Q

		NA[d]							M1			NS1				NS2	
		49 (48)	72 (75)	166 (169-170)	272 (275)	289 (292)	300 (303)	306 (309)	165	205	236	80	180	216	218	60	115
Guangdong2016	Human	E	M	Y	I	**R**	I	**T**	I	I	N	S	I	T	-	S	T
Taiwan2017		G	I	H	V	K	V	A	M	I	H	T	V	P	K	K	**T**
Consensus sequence	Chicken	E	M	Y	I	R/K	I	A	M	V	N	S	I	T	-	S	T/D/-

[a]Bold font denotes the substituted residue after egg passage, PB2: N82K; HA1: G137E; HA2: Q42R; NA: K289R and A306T; NS2: L115T

[b]Amino acids consensus sequence in 24 HPAIVs of H7N9 subtype isolated from chickens

[c]Numbers in parentheses represent the positions in H3 numbering

[d]Numbers in parentheses represent the positions in N2 numbering

strains of HPAIVs isolated before 2014 [40]. These results indirectly suggest that H5 HPAIVs have become adapted to migratory birds, enabling them to replicate well in wild birds over long periods. Thus, such adaptation of H7N9 HPAIVs to migratory birds could be possible in the future, facilitating their global dissemination. In this study, we demonstrated the preferential replication and the high pathogenicity of H7N9 HPAIVs isolated from humans in chickens. They did not replicate in and were not shed efficiently from domestic ducks, an observation that is important for epidemic control strategies for H7N9 HPAIVs.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical standard statement All experimental and animal procedures were approved by the ethics committee of the National Institute of Animal Health, Japan.

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