Supplementary data

Genetic characterization of avian influenza A (H11N9) virus isolated from Mandarin duck in South Korea in 2018

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Fig. S1. Sampling location of A/Mandarin duck/South Korea/KNU18-12/2018(H11N9).

BOLD TaxonID Taxonomy Report

Order

Family

Species

Anseriformes[99 individuals][7 species]

Anatidae[99 individuals][7 species]

Aix galericulata[24 individuals] Aix sponsa[9 individuals] Anas crecca[59 individuals] Cairina moschata[3 individuals] Melanitta nigra[1 individuals] Somateria fischeri[2 individuals]

Fig.

Fig. S2. Information of bird species identification

Table S1. Initial input of barcoding

TAATCTTCTTCATGGTGATACCCATCATAATTGGAGGATTCGGCAATTGACTAGTCC CCCTAATAATTGGCGCCCCTGACATGGCATGCCCCGAATGAACAACATAAGCTTCT GACTCCTTCCACCCTCATTCCTCCTACTGCTCGCCTCATCTACCGTGGAAGCTGGCGC CGGTACAGGCTGAACCGTGTACCCACCCCTAGCTGGCAACCTAGCCCACGCCGGAG CCTCAGTAGACCTAGCCATCTTCTCACTCCACTTAGCCGGTGTTTCCTCCATCCTCGG AGCCATTAACTTCATTACTACGGCCATCAACATAAAACCTCCCGCACTCTCACAATA CCAAACTCCACTCTTCGTCTGATCCGTCCTAATTACTGCCATCCTACTCCTCCTGTCC CTCCCCGTTCTTGCCGCTGGCATCACAATGCTACTAACTGACCGAAACCTAAACACC ACATTCTTCGACCCGCCGGAGGAGGAGACCCAATCCTGTATCAACACCTA



Fig. S3. Location map of Mandarin ducks marked with satellite transmitters in the East Asian Flyways, March and October in 2018. Red dot indicate the isolation place of A/Mandarin duck/South Korea/KNU18-12/2018(H11N9) and blue dots indicate the position of mandarin duck. Map was retrieved from National Institute of Biological Resources, Korea.

Table S2. Detailed NGS analysis

Sample		Sequenced Sample						R_ORF		S_ORF																
San le Seg ent	1p m	ge ne na m e	# of Pre- pro cess ed rea ds	# of Infl uen za Vir us extr acte d rea ds	# of non - Infl uen za Vir us rea ds	Vir us rea ds %	#M_ Rea ds	Uni que Ma tch es	S_C on bp	%Co v.(S/ R)	Le ng th	S_p ositi on	E_p ositi on	Le ng th	S_p ositi on	E_p ositi on	met hod	R_ OR F - S_ OR F len gth	Co nti g Co un t							
	1	Р В 2					1714 8	0.0 29	2292	1	22 80	1	2280	22 74	19	2292		6	1							
	2	P B 1, P B 1- F2												1799 9	0.0	2280	1	22 74	1	2274	22 74	3	2276	stan	0	1
K 10 2	3	P A, P A- X	232 636 54	218 668 8	210 769 66	0.1 037 48	1637 2	0.0 27	2151	1	21 51	1	2151	21 51	1	2151	dar d :seg me nt 8	0	1							
	4	H A					5330 3	0.0 89	1688	1	16 89	1	1689	16 89	1	1689		0	1							
	5	N P					9701 9	0.1 62	1505	0.99	14 97	1	1497	15 06	1	1506		-9	1							
	6	N A					4713	0.0 08	1419	0.979 5	14 13	1	1413	13 95	5	1399		18	1							
	7	M 2,					3335 64	0.5 56	980	0.999	75 9	1	759	75 9	1	759		0	1							

	M 1														
8	N E P, N S1			3532 9	0.0 59	822	0.972 8	69 3	1	693	69 0	2	691	3	1

Raw sequence reads were quality trimmed using "trim_galore"(q=20) and non-influenza virus read was removed using Deconseq (iden=60) and python script was used as tool to adjust the amount of data to up to 600,000 read. Meanwhile a database of only segment 4 (HA), 6 (NA), and 8 (NS1) from the Influenza virus that NCBI was created to provide and align to those of the reference using Gsmapper. (iden=70, ml=40).

ORF was observed with the consensus obtained and adopted a result with an ORF similar to the reference.

As ORF length was different from the reference, sequence error was corrected using ProovRead as previously described ¹.

In the NGS reading result, influenza virus reading showed 33.04% reading (influenza: no-influenza = 6404260:19381530) rate and all ORFs were completed.

Gene	A/Mandarin duck/South Korea/KNU18-12/2018(H11N9) (GenBank accession # ^a)	Virus with the highest homology (GenBank ccession No. ^b)	Nucleotide identity ^c (%)	% Nucleotide identity ^d (%)
PB2	MN596421	A/mallard/Khabarovsk/241/2017 (H10N6)(EPI1333694)	98.9	94.53
PB1	MN596422	A/wild bird/Eastern China/1754/2017(H5N3)(MN171456.1)	99.08	92.31
РА	MN596423	A/duck/Jiangsu/SE0261/2018(H5N3) (MN171449.1)	99.21	96.72
НА	MN596424	A/duck/Kagoshima/KU57/2014 (H11N9)(EPI1057756) A/crane/Kagoshima/KU-T40/2015 (H11N9)(EPI1057748)	- 97.70	94.99
NP	MN596425	A/duck/Aichi/231003/2016 (H8N4)(EPI866835)	99.35	97.26
NA	MN596426	A/duck/Kagoshima/KU57/2014 (H11N9)(EPI1057758)	98.00	94.69
Μ	MN596427	A/wild waterfowl/Korea/F7-18/2018 (H4N8)(EPI1566658)	99.80	98.40
NS	MN596428	A/avian/Japan/8KI0162/2008 (H3N8)(EPI299270)	99.54	74.89

 Table S3. Homology analysis of each gene of A/Mandarin duck/South Korea/KNU18-12/2018(H11N9)

^a GeneBank Accession number of eight gene segments of A/Mandarin duck/South Korea/KNU18-12/2018(H11N9) ^b GeneBank Accession number listed in NCBI and GISAID database

^c Nucleotide identity of virus showing the highest homology

^d Nucleotide identity of A/waterfowl/Korea/S353/2016 (H11N9)

a. H1N1 (12 hpi)



b. H1N1 (24 hpi)



c. H1N1 (36 hpi)

d.H1N1 (48 hpi)





g. H11N9 (12 hpi)



h. H11N9 (24 hpi)





k. H11N9 (60 hpi)



m. H9N2 (12 hpi)



n. H9N2 (24hpi)

p. H9N2 (48 hpi)



q. H9N2 (60 hpi)



r. H9N2 (72 hrs)



Fig. S4. Raw ELISA data to conduct $TCID_{50}$ assay. Serial 10-fold dilutions of three different viruses H1N1 (a-f), H11N9 (g-l), and H9N2 (m-r) (MOI of initial virus stock: 0.001) infected MDCK for 3 dpi. At every 12 hours post-infection (hpi), cell monolayers were fixed with 10% acetone and blocked with 5% non-fat milk. Cells were washed with PBS and reacted with 0.1 µg/well of anti-influenza nucleoprotein (Medix Biochemica, Finland). After 1 h, secondary Ab in the form of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Abcam, Cambridge, UK) was added to each well according to the manufacture's protocol. Stringent washing with PBS-T was performed five times to remove

nonspecific biding and 100 μ L of 3,3',5,5'-tetra methyl benzidine (Sigma-Aldrich) substrate solution was added. 0,10⁰- fold dilution of stock; 1,10¹- fold dilution of stock; 2,10²- fold dilution of stock; 3,10³- fold dilution of stock; 4,10⁴- fold dilution of stock; 5,10⁵- fold dilution of stock; 6,10⁶- fold dilution of stock; 7,10⁷- fold dilution of stock; 8,10⁸- fold dilution of stock; 9,10⁹- fold dilution of stock; 10,10¹⁰- fold dilution of stock; C, mock-infection;



H11N9 - 14 DPI H11N9 - 14 DPI H11N9 - 14 DPI

Fig. S5. Raw ELISA data to conduct TCID₅₀ assay to measure virus titer in lung. H1N1 (a) and H11N9 (b). $0,10^{0}$ - fold dilution of stock; $1,10^{1}$ - fold dilution of stock; $2,10^{2}$ - fold dilution of stock; $3,10^{3}$ - fold dilution of stock; $4,10^{4}$ - fold dilution of stock; $5,10^{5}$ - fold dilution of stock; $6,10^{6}$ - fold dilution of stock; $7,10^{7}$ - fold dilution of stock; $8,10^{8}$ - fold dilution of stock; $9,10^{9}$ - fold dilution of stock; $10,10^{10}$ - fold dilution of stock; C, mock-infection;

Countr		Nucleotide identity									
У	HIIN9 Strains	PB2	PB1	РА	НА	NP	NA	М	NS		
	A/mallard/Sanjiang/148/20 06	92.81 %	95.26 %	96.05 %	95.35 %	92.59 %	95.61 %	96.84 %	_*		
	A/Oriental White Stork/Zhalong/183/2006	92.68 %	95.21 %	95.40%	95.17 %	91.84 %	95.75 %	96.74 %	-		
	A/wild bird/Anhui/S119/2014	97.68 %	95.74 %	95.03 %	95.17 %	97.66 %	94.83 %	97.15 %	94.58 %		
	A/wild bird/Anhui/L306/2014	88.13 %	96.09 %	90.00 %	95.35 %	93.99 %	94.90 %	98.27 %	90.80 %		
China	A/wild bird/Wuhan/CDHN01/2015	95.48 %	95.22 %	95.53%	95.24 %	97.66 %	94.84 %	98.70 %	75.11 %		
	A/wild bird/Wuhan/CDHN22/2015	95.48 %	95.22 %	95.53%	95.24 %	97.60 %	94.84 %	98.70 %	75.11 %		
	A/wild bird/Wuhan/CDHN173/201 5	95.48 %	95.22 %	95.53%	95.24 %	97.54 %	94.84 %	98.70 %	75.11 %		
	Bean goose/Hubei/SZY200/2016	97.63 %	95.25 %	96.98%	80.78 %	97.26 %	94.48 %	97.96 %	-		
	A/duck/Niigata/151015/201 6	95.66 %	96.20 %	94.85 %	95.13 %	97.91 %	94.53 %	96.40 %	94.32 %		
	A/duck/Niigata/151019/201 6	95.62 %	96.20 %	94.85 %	95.07 %	97.91 %	94.46 %	96.40 %	94.32 %		
	A/duck/Niigata/151014/201 6	95.66 %	96.20 %	94.85 %	95.13 %	97.91 %	94.53 %	96.40 %	94.32 %		
T	A/duck/Ibaraki/99/2016	88.04 %	95.19%	98.68 %	95.13 %	91.91 %	94.88 %	95.50 %	78.12 %		
Japan	A/duck/Ibaraki/F99/2016	88.04 %	95.24 %	98.68 %	95.13 %	91.84 %	94.88 %	95.50 %	74.91 %		
	A/crane/Kagoshima/ KU- T40/2015	96.01 %	93.80 %	96.79 %	97.70 %	91.45 %	97.95 %	99.39 %	-		
	A/duck/Kagoshima/KU57/2 014	95.96 %	93.85 %	96.79 %	97.70 %	91.38 %	98.02 %	99.39 %	-		
	A/duck/Tottori/311217/201 4	97.83 %	87.41 %	95.26 %	95.43 %	97.39 %	94.98%	96.45 %	90.60 %		
Korea	A/waterfowl/Korea/S353/2 016	94.53 %	92.31%	96.72%	94.99 %	97.26 %	94.69 %	98.40 %	74.89 %		
	A/ruddy turnstone/ Ilha de Canelas/A008/2008	84.60%	88.16 %	89.62%	80.94%	88.78 %	87.17 %	93.71 %	77.65%		
Brazil	A/ruddy turnstone/Ilha de Canelas/A017/2008	84.73 %	88.02%	89.62%	80.88 %	88.85 %	87.31 %	93.61 %	77.81 %		
	A/ruddy turnstone/Ilha de Canelas/A051/2008	84.72 %	88.11 %	89.62%	81.00 %	88.85 %	87.24 %	93.71 %	77.97 %		

Table S4. Genetic similarity with H11N9 strains of different countries

* No similarity

Table S5. Stock titer of virus used in mouse study

Virus	Characteristics							
v nus	EID ₅₀ /mL	TCID ₅₀ /mL	PFU/mL					
KH1N1	10 ^{5.17}	$10^{4.67}$	10 ^{4.52}					
H11N9	10 ^{7.18}	10 ^{6.2}	10 ^{6.05}					

Gene	Known mouse adaptive	A/California/04/2009(H1N1)
	mutation site ³	
PB2	E158G/A	E158
PA	L295P	295P*
NP	D101G	D101
	H289Y	H289
HA	K119N	K119 (136)
	G155E	G155 (172)
	S183P	183P (200)*
	R221K	R221 (238)
	D222G	222G (239)*

Table S6. Mouse adaptive mutation site of A/California/04/2009 (H1N1)

*: Mutation site of A/California/04/2009(H1N1)



Fig. S6. Close relationship of PA gene of different isolates in Korea in 2018. A/duck/Jiangsu/SE0261/2018 (H5N3) showed the close relation with two different isolates (A/Mandarin duck/South Korea/KNU18-12/2018(H11N9) and A/wild duck/Korea/K102/2018(H2N9)².

Reference

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2. Yeo, S. J.; Than, D. D.; Park, H. S.; Sung, H. W.; Park, H., Molecular Characterization of a Novel Avian Influenza A (H2N9) Strain Isolated from Wild Duck in Korea in 2018. *Viruses* **2019**, *11* (11).

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