Replication of Novel Zoonotic-Like Influenza A(H3N8) Virus in Ex Vivo Human Bronchus and Lung

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Human infection with avian influenza A(H3N8) virus is uncommon but can lead to acute respiratory distress syndrome. In explant cultures of the human bronchus and lung, novel H3N8 virus showed limited replication efficiency in bronchial and lung tissue but had a higher replication than avian H3N8 virus in lung tissue.

Avian influenza viruses (AIVs) with reassortments between AIVs from domestic poultry and wild birds sporadically cross species barriers, leading to human infections. Viruses with internal genes of H9N2, hemagglutinin, and neuraminidase acquired from wild birds constitute the zoonotic H5N1, H7N9, and H10N8 viruses (*1*–*3*) and can lead to severe influenza.

In 2022, two human infections with novel influenza A(H3N8) viruses were reported in Henan and Hunan Province, China (*4*,*5*). The first case was identified in a 4-year-old boy with acute respiratory distress syndrome, and the second case occurred in a 5-year-old boy with mild disease. Phylogenetic analysis revealed that the novel H3N8 viruses were triple reassortments containing the Eurasian avian H3 gene of wild-bird origin, the North American avian N8 gene derived from the wild bird AIV, and G57 genotype H9N2 internal genes from AIVs found in poultry in China (*6*,*7*). H3N8 viruses that are genetically similar to the zoonotic H3N8 viruses reported in China (*4*,*5*) have been isolated in poultry markets in Hong Kong, China (*8*). Those novel avian H3N8 viruses are antigenically distant from contemporary human influenza A(H3N2) viruses, and little cross-reactive immunity to these chicken H3N8 viruses exists in the human population (*8*). We assessed the replication of the novel influenza A(H3N8) virus in human ex vivo bronchus and lung tissues (Appendix, https://wwwnc.cdc.gov/EID/article/29/6/22-1680-App1.pdf).

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The Study

The viruses used in this study were H9N2/Y280, pH1N1, avH3N8/MP16, novel H3N8, and H5N1/483 (Appendix Table 1). The novel H3N8 virus was isolated from chickens and is genetically closely related to the virus causing zoonotic human disease in China (A/Henan/4-10CNIC/2022/H3N8) (*8*). Their hemagglutinin genes share a 99.1% similarity, and the neuraminidase genes share a 98.7% similarity. The avH3N8 virus was isolated from wild bird droppings in Mai Po, Hong Kong, and is genetically unrelated to the virus causing zoonotic disease in China. The novel H3N8 virus failed to propagate in Madin-Darby canine kidney (MDCK) cells but could be propagated in eggs and titrated in chicken embryo fibroblasts (DF-1), whereas the other strains could be propagated and titrated in MDCK cells. We performed titration in cells that support the replication of the influenza A viruses rather than in all DF-1 cells, because pH1N1 virus did not replicate in DF-1 cells. We investigated the virus replication kinetics by measuring viral matrix protein segment RNA in culture supernatants using real-time quantitative reverse transcription PCR and 50% tissue culture infectious dose ($TCID_{50}$) assay for infectious virus titers (Figure 1).

In bronchial tissues, pH1N1 virus had a higher level of viral RNA than did avH3N8, novel H3N8, and H5N1, whereas levels of viral RNA of H9N2 virus were higher than those of avH3N8 and H5N1 virus (Figure 1, panels A, C). The viral RNA levels of avH3N8, novel H3N8, and H5N1 virus were similar.

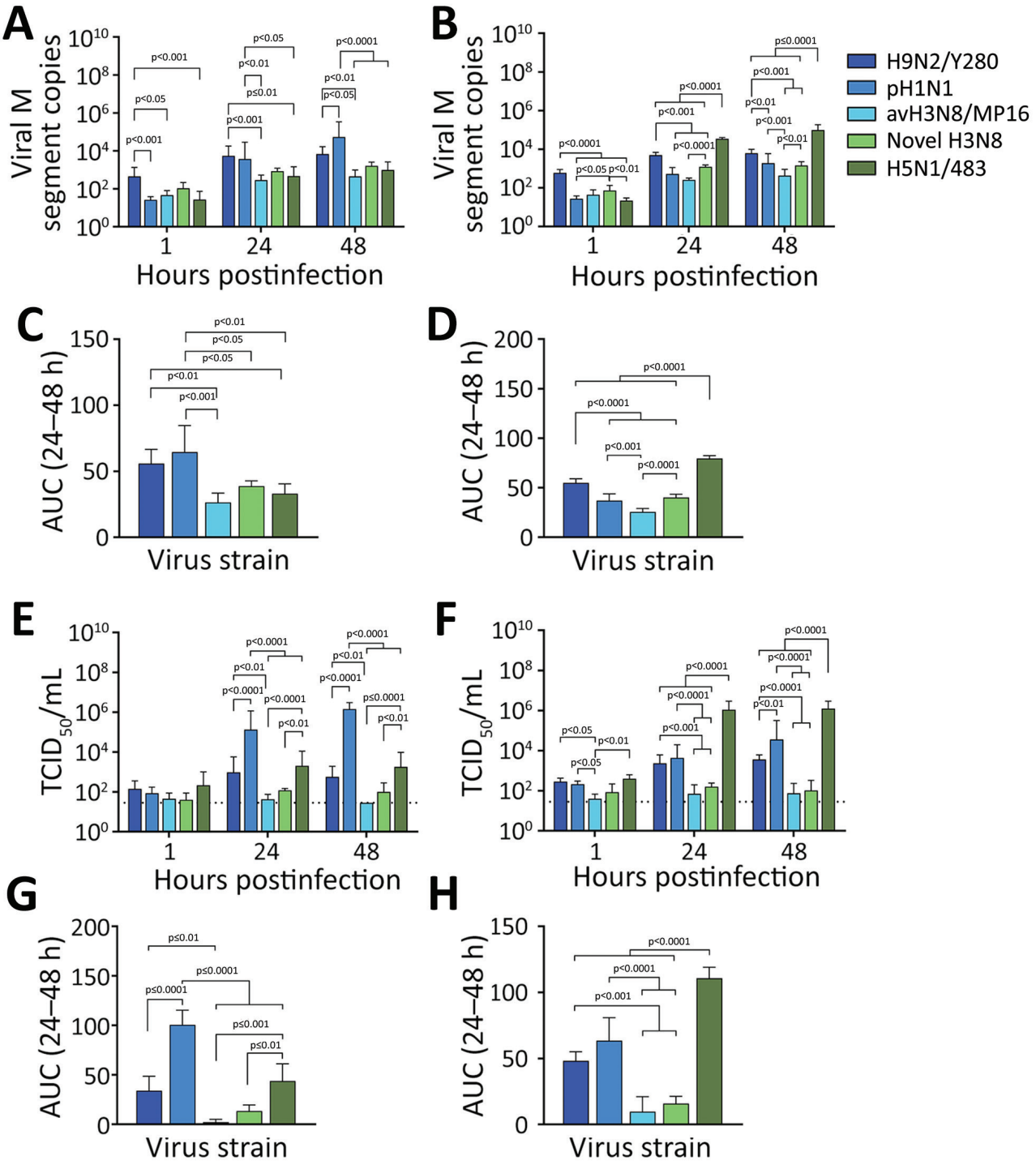

Figure 1. Comparative replication competence of zoonotic-like influenza A(H3N8) viruses isolated from chicken and other human and avian viruses in ex vivo cultures of human bronchus and lung tissue. Viral M segment RNA copies (A, B) and viral titers (E, F) in culture supernatants were collected at 1, 24, and 48 hours postinfection with H9N2/Y280, pH1N1, avH3N8/MP16, novel H3N8, or H5N1/483 viruses and measured by quantitative reverse transcription PCR (A, B) and $TCID_{50}$ (E, F). C, D) Viral load from panels A and B by virus strain. G, H) Viral titers from panels E and F by virus strain. Data are geometric mean ±SD. Statistical analysis was performed using 2-way (A, B, E, F)or 1-way (C, D, G, H) analysis of variance followed by Tukey posttest; $p<0.05$ was considered to be statistically significant. Detailed information on viruses used in study is provided in the Appendix (https://wwwnc.cdc.gov/EID/article/29/6/22-1680-App1.pdf). AUC, area under the curve; M, matrix; $TCID_{50}$, 50% tissue culture infectious dose.

The viral RNA level of H5N1 virus was the highest among all the tested strains in human lung tissues, followed by H9N2 (Figure 1, panels B, D). Viral RNA levels of novel H3N8 and pH1N1 viruses were higher than those of avH3N8 virus. Measurement of viral RNA using quantitative reverse transcription PCR is sensitive, but it cannot distinguish defective viral particles from infectious ones. Therefore, we performed the $TCID_{50}$ assay to monitor the infectious viral titers.

As expected, replication of pH1N1 virus was the highest among all tested strains in human bronchial tissues, in both viral titers and area under the curve values (Figure 1, panels E, G). The titers of H5N1 virus were similar to those of H9N2 virus, whereas H5N1 virus had higher replication competence than did avH3N8 and novel H3N8 viruses. We observed a discrepant trend between viral RNA copies and infectious titers for H9N2. Viral RNA levels of H9N2 were similar to those of pH1N1 (Figure 1, panels A, C), but the infectious titers of H9N2 virus were significantly lower than that of pH1N1 in bronchus (Figure 1, panels E, G).

In lung tissue, H5N1 virus had the highest replication of all strains tested (Figure 1, panels F, H). Similar to pH1N1 virus, H9N2 had higher titers than the 2 H3N8 viruses in lung tissues. avH3N8 had the lowest titer measured by $TCID_{50}$. The novel H3N8 virus replicated poorly in mammalian MDCK cells but replicated efficiently in DF-1 avian cells (Appendix Figure). Those findings imply that the novel H3N8 virus has not yet adapted to mammal hosts, which was confirmed by limited replication in human bronchial and lung tissues (Figure 1, panels E, F).

We fixed infected tissues at 48 hours postinfection and stained them for influenza A nucleoprotein immunohistochemistry (Appendix). Consistent with $TCID_{50}$ findings, bronchial tissues infected with pH1N1 had the most extensive distribution of viral

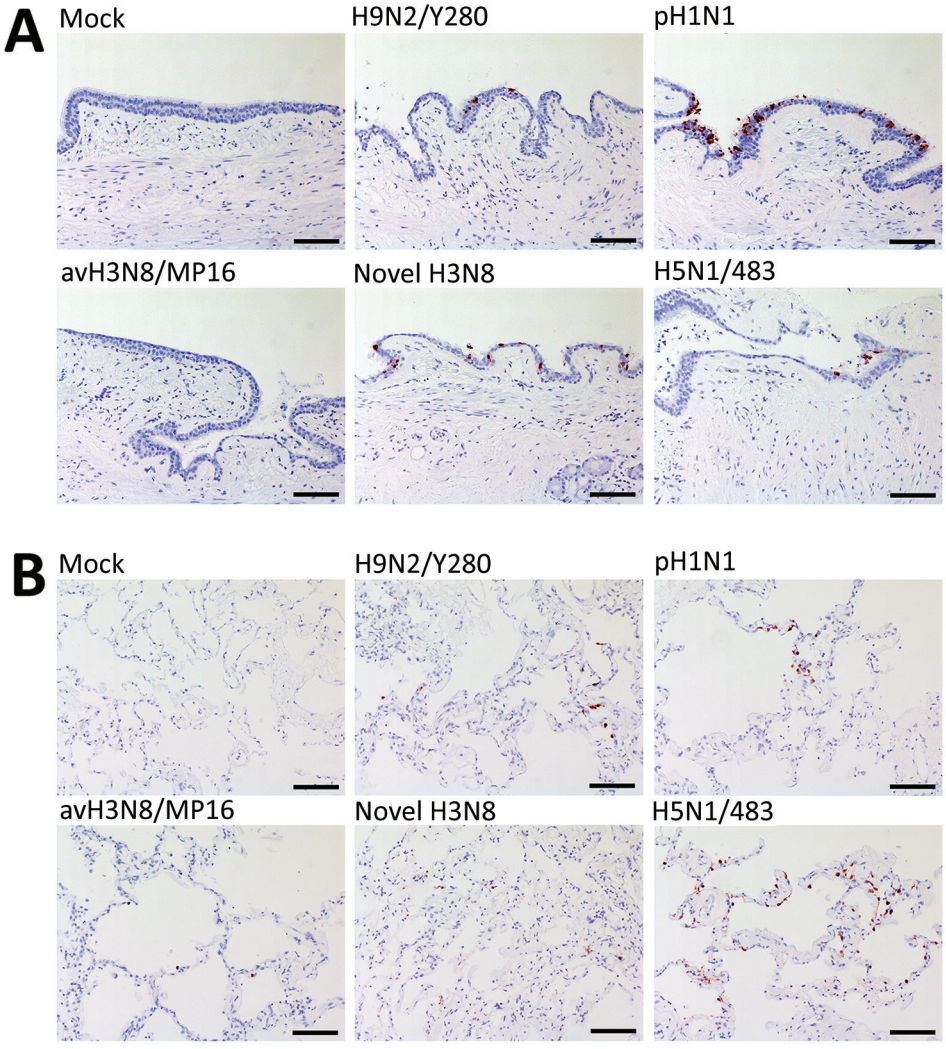

Figure 2. Tissue tropism of influenza A viruses in ex vivo cultures of human bronchus and lung tissue. Immunohistochemical staining of influenza A nucleoprotein in ex vivo cultures of human bronchial tissues (A) and lung tissues (B) at 48 hours postinfection with H9N2/Y280, pH1N1, H3N8/MP16, novel H3N8, and H5N1/483 viruses. Positive cells are indicated by red-brown color. Images are representative of 3 individual donors. Scale bar indicates 100 μm. Detailed information on viruses used in study is provided in the Appendix (https://wwwnc.cdc.gov/EID/article/29/6/22-1680-App1.pdf).

Table. Source of gene segments of novel and avian influenza A(H3N8) viruses*

Gene segment	Novel H3N8	avH3N8/MP16
Polymerase basic 2	H9N2	H3N8
Polymerase basic 1	H9N2	H6N1
Polymerase acidic	H9N2	H6N2
Hemagglutinin	H3N8	H3N8
Nucleoprotein	H9N2	H3N8
Neuraminidase	H3N8	H3N8
Matrix	H9N2	H1N1
Nonstructural	H9N2	H7N1

*Detailed information on viruses used in study is provided in the Appendix (https://wwwnc.cdc.gov/EID/article/29/6/22-1680-App1.pdf).

antigen, whereas we observed moderate levels of viral antigen staining in tissues infected with novel H3N8, H9N2, and H5N1 virus. No viral antigen staining was observed in tissues infected with avH3N8 (Figure 2, panel A). In the lung, H5N1 virus infection demonstrated the most extensive viral antigen staining, followed by infection with pH1N1, novel H3N8, and avH3N8 virus, which demonstrated the least extensive staining (Figure 2, panel B).

The discrepancy between the viral load in RNA copies and $TCID_{50}$ titers of H9N2 and avH3N8 infection suggests that infection with those viruses might produce high levels of defective particles that cannot be detected by $TCID_{50}$ assay. Immunohistochemistry staining of viral antigen serves as alternative evidence of virus replication in human tissues. The staining correlates more with $TCID_{50}$ results than with viral RNA analysis for all the viruses.

Amino acid comparisons of the novel H3N8 and avH3N8 viruses demonstrated that they shared the same stalk length in the NA gene but did not have the G228S mutation that enhances binding to mammalian receptors (Appendix Table 2). The internal genes of the novel H3N8 virus were reassorted from H9N2 virus, whereas the internal genes of the avH3N8 came from H3N8, H6N1, H6N2, H3N8, H1N1, and H7N1 (Table). Neither virus had the E627K mutation in polymerase basic 2 that confers mammal adaptation, virulence, and transmissibility. The novel H3N8 virus had the A588V mutation in polymerase basic 2 that promotes mammal adaptation, but avian H3N8 virus did not have this mutation. This difference might contribute to higher replication of the novel H3N8 virus in human lung tissue. The S31N mutation found in the matrix protein 2 of the novel H3N8 virus provided adamantane resistance.

Conclusions

Although zoonotic H3N8 viruses have a dual receptor-binding affinity of α-2,3 and α-2,6 receptors (*7*), our findings show that this factor does not confer an advantage for replication in human bronchial tissue. Our findings demonstrated inefficient replication of the novel H3N8 virus in human bronchial tissues, which implies limited efficiency to transmit among humans. This finding is in line with a recent serologic surveillance study in which no poultry workers were positive for antibodies for the novel H3N8 virus (*7*), and only 2 human cases have been documented since April 2022 (*4*,*5*). The moderate replication ability of the novel H3N8 virus in human lung tissue suggests that the virus causes less severe disease than H5N1 virus.

In summary, our findings suggest that the zoonotic-like avian H3N8 virus has limited efficiency for human-to-human transmission and, at present, is unlikely to cause severe disease in humans. However, the limited cross-reactive immunity against this novel H3N8 virus in the human population (*8*) and the emergence of novel H3N8 viruses by continuous reassortment between AIVs in wild birds and poultry demonstrate that the zoonotic and pandemic potential of avian H3N8 viruses should be closely monitored.

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Dr. Hui is an assistant professor in the School of Public Health at the University of Hong Kong. Her research interests include risk assessment, understanding the pathogenesis of emerging respiratory viruses, and the development of therapeutic options for severe influenza diseases and coronavirus infections.

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Appendix

Materials and Methods

Viruses

In addition to the zoonotic-like influenza H3N8 (A/Env/Hong_Kong/MKT_AB_13cp/2022, novel H3N8) which is a chicken virus, an avian virus isolated from wild bird droppings in Mai Po, Hong Kong (A/Env/Hong Kong/MP16_265/2016, avH3N8/MP16), a highly pathogenic avian influenza (HPAI) H5N1 virus (A/Hong Kong/483/1997, H5N1/483) isolated from a fatal human infection, a 2009 pandemic influenza virus (A/Hong Kong/415742/2009, pH1N1) isolated from a patient in Hong Kong and a duck H9N2 virus (A/Duck/Hong Kong/Y280/97, H9N2/Y280) were used as controls. The novel H3N8 virus was propagated in eggs while all the other influenza viruses were passaged in Madin-Darby canine kidney (MDCK) cells. Virus stock was aliquoted and titrated to determine the plaque forming unit per mL (pfu/mL) in chicken embryo fibroblast (DF-1) cells for the novel H3N8 virus and MDCK cells for the other influenza viruses. The experiments were carried out in a Bio-safety level 3 (BSL-3) facility at the School of Public Health, LKS Faculty of Medicine, The University of Hong Kong.

Ex vivo cultures and infection of human bronchus and lung

Fresh non-tumor bronchus and lung tissues were obtained from patients undergoing elective surgery in Department of Surgery of Queen Mary Hospital and were removed as part of clinical care but surplus for routine diagnostic requirements as detailed previously (*1*). Sampling

of tissues is defined by convenience. Fragments of human tissues were infected with each virus at $1x10^6$ pfu/mL for 1 h at 37°C. Mock-infected tissue served as negative controls. The explants were washed three times with PBS and placed in culture medium (F-12K nutrient mixture with L-glutamine, and antibiotics) with or without a sterile surgical pathology sponge to establish an air-liquid interface condition in 24-well culture plates in a 37°C incubator with 5% CO_2. Infectious viral titers in culture supernatants were assessed at 1, 24, and 48 hours post-infection (hpi) by $TCID_{50}$ assay. Bronchus and lung tissues were fixed at 48 hpi in 10% formalin and processed for immunohistochemistry staining. Ethics approval of the use of human tissues was granted by the institutional review board of the University of Hong Kong and the hospital authority (approval number UW 20–862).

Viral titration by $TCID_{50}$ assay

A confluent 96-well tissue culture plate of DF-1 or MDCK cells was prepared 1 day before the virus titration ($TCID_{50}$) assay. Cells were washed once with PBS and replenished serum-free MEM medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and 2 μg/ml of tosylsulfonyl phenylalanylchloromethyl ketone treated trypsin for DF-1 and MDCK cells. Serial dilutions of virus supernatant, from 0.5 log to 7 log, were performed before adding the virus dilutions onto the plates in quadruplicate. The plates were observed for cytopathic effect daily. The endpoint of viral dilution leading to CPE in 50% of inoculated wells was estimated using the Karber method (*2*). AUC was calculated from the viral titers from different time points indicated in the y-axis.

Quantitative reverse transcription PCR assay

The viral RNA in 20 uL culture supernatants was extracted using the QIAamp Viral RNA Mini kit (Qiagen). RNA was reverse-transcribed by using random 6-mer primers with PrimeScript RT reagent Kit (Takara). Viral RNA copy number of target genes was performed using an ABI ViiA 7 real-time PCR system (Applied Biosystems). All procedures were performed according to the manufacturers' instructions as previously described (*3*,*4*).

Immunohistochemical staining

The human tissues were fixed with 10% formalin overnight, embedded in paraffin blocks and stained for influenza viral protein as previously described (*4*). Briefly, the paraffin-embedded tissues were sectioned, deparaffinised, digested with pronase and blocked with an

avidin/biotin blocking kit (Vector Labs, Burlingame, CA, USA). The sections were then stained with influenza A virus nucleoprotein-specific mouse monoclonal antibody HB65 (EVL, Woerden, The Netherlands) and a biotinylated secondary antibody. The bound antibodies were visualized with a Strep-ABC complex and an AEC substrate kit.

Statistical analysis

Experiments with the human ex vivo cultures were performed independently with five and six different donors of bronchus and lung, respectively. Results shown in figures are geometric mean (+/−SD). Area-under-the-curve (AUC) was calculated by integrating infectious virus titers at 24–48 hpi in ex vivo bronchus, lung tissues. The differences in log10-transformed viral titers and quantitative viral RNA of M segment between viruses and over time were compared using two-way ANOVA followed by a Tukey's multiple-comparison test using GraphPad Prism version 9.0.0. Comparison of AUC between viruses was calculated using one-way ANOVA followed by a Tukey's multiple-comparison test. Differences were considered significant at a *P* value less than 0.05.

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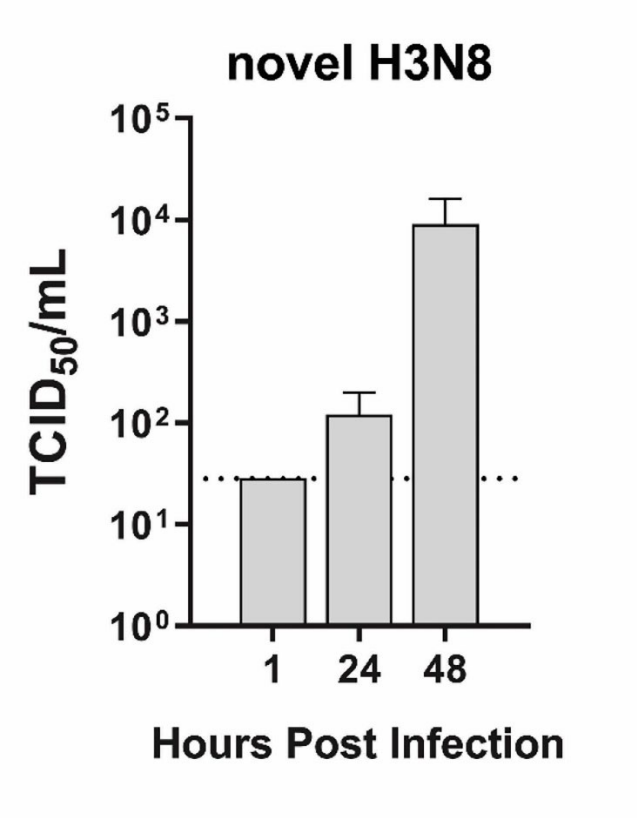

Appendix Figure. Novel H3N8 virus replication in DF-1 cells infected at MOI 0.01. $TCID_{50}$ assay was performed in DF-1 cells. Dotted line represents the detection limit of $TCID_{50}$ assay.

Appendix Table 1. Influenza A virus strains used

Virus	Strain name
H9N2/Y280	A/Duck/Hong Kong/Y280/97
pH1N1	A/Hong Kong/415742/2009
avH3N8/MP16	A/Env/Hong Kong/MP16_265/2016
Novel H3N8	A/Env/Hong_Kong/MKT_AB_13cp/2022
H5N1/483	A/Hong Kong/483/1997

Appendix Table 2. Amino acid comparison between the novel H3N8 (AB_13) and avian H3N8 (MP16)

HA1	amino acid position	2	6	8	19	45	57	58	70	83	92	102	112	119	124	128	131	137	145	159
A/Env/Hong_Kong/MKT_AB_13cp/2022		N	K	S	S	N	K	V	M	K	S	V	I	D	S	A	S	G	N	S
A/Env/Hong_Kong/MP16_265/2016_(H3N8)_Anas_acuta		D	N	N	A	S	R	I	L	T	N	I	V	E	G	T	T	S	S	N
	amino acid position	160	163	172	214	228	257	260	264	275	312									
		S	L	Y	V	G	F	I	R	E	N									
		A	V	D	I	G	Y	M	K	D	T									

NA	amino acid position	14	20	22	26	30	37	38	39	43	44	49	50	59	61	62	69	71	74	75
A/Env/Hong_Kong/MKT_AB_13cp/2022		V	L	I	I	T	P	R	D	R	N	V	I	I	K	V	N	I	I	E
A/Env/Hong_Kong/MP16_265/2016_(H3N8)_Anas_acuta		I	F	V	V	I	G	K	G	G	V	I	V	V	R	I	S	V	M	P
	amino acid position	76	77	78	79	80	81	82	89	92	125	154	161	191	198	209	235	237	253	254
		R	P	E	N	D	H	F	L	A	T	Y	I	I	A	I	E	F	A	F
		Y	W	K	E	G	T	Y	i	V	I	F	V	V	S	V	D	Y	I	Y
	amino acid position	257	260	263	265	266	267	283	311	329	355	375	376	377	381	383	388	389	392	397
		K	K	E	A	E	I	E	R	S	N	K	V	R	V	N	I	K	V	S
		S	R	G	R	D	V	D	K	A	T	R	I	K	T	T	V	R	I	L
	amino acid position	410	414	415	416	452	469													
		V	K	R	N	I	E													
		I	G	K	D	V	K													

PB2	amino acid position	588	627
A/Env/Hong_Kong/MKT_AB_13cp/2022		V	E
A/Env/Hong_Kong/MP16_265/2016_(H3N8)_Anas_acuta		A	E

M2	amino acid position	31
A/Env/Hong_Kong/MKT_AB_13cp/2022		N
A/Env/Hong_Kong/MP16_265/2016_(H3N8)_Anas_acuta		S