
Risk Assessment for Highly Pathogenic Avian Influenza A(H5N6/H5N8) Clade 2.3.4.4 Viruses

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The numerous global outbreaks and continuous reassortments of highly pathogenic avian influenza (HPAI) A(H5N6/H5N8) clade 2.3.4.4 viruses in birds pose a major risk to the public health. We investigated the tropism and innate host responses of 5 recent HPAI A(H5N6/H5N8) avian isolates of clades 2.3.4.4b, e, and h in human airway organoids and primary human alveolar epithelial cells. The HPAI A(H5N6/H5N8) avian isolates replicated productively but with lower competence than the influenza A(H1N1)pdm09, HPAI A(H5N1), and HPAI A(H5N6) isolates from humans in both or either models. They showed differential cellular tropism in human airway organoids; some infected all 4 major epithelial cell types: ciliated cells, club cells, goblet cells, and basal cells. Our results suggest zoonotic potential but low transmissibility of the HPAI A(H5N6/H5N8) avian isolates among humans. These viruses induced low levels of pro-inflammatory cytokines/chemokines, which are unlikely to contribute to the pathogenesis of severe disease.

The genetic evolution of the highly pathogenic avian influenza (HPAI) subtype H5N1 A/goose/Guangdong/1/1996 lineage has resulted in the divergence and generation of 10 distinct virus clades (0–9) and multiple subclades (1,2). Since early 2014, novel reassortant HPAI A(H5N6/H5N8) viruses of clade 2.3.4.4 have gained attention because of their rapid evolution and global spread. They have been widely distributed among regions of Asia, Europe, and Africa and have been reported in North America (mainly in the United States and Canada), accompanied

by further evolution into subclades 2.3.4.4a–h (3). Many avian species, including wild aquatic birds, domestic poultry, and zoo birds, are susceptible to the infection or support transmission of clade 2.3.4.4 viruses, resulting in unprecedented panzootic waves accompanied by massive culling and major economic losses to the poultry industry (1,2). Detection of HPAI H5N6 clade 2.3.4.4 viruses in cats, pigs, and humans has also been reported (4–6). As of May 2021, there have been 29 laboratory-confirmed cases of human infection, including at least 16 deaths (3,6–8). The isolated viruses mainly belong to subclades 2.3.4.4a, b, d, g, and h. These cases in humans were mainly sporadic and linked to direct contact with poultry or contaminated poultry market environments (7). The initial clinical signs in hospitalized patients were influenza-like, followed by severe pneumonia, acute respiratory distress syndrome, and multiple organ failure in deceased patients (7,8). Natural infection of mammals by HPAI H5N8 clade 2.3.4.4 viruses is not as commonly reported.

In February 2021, the Russian Federation reported detecting 7 cases of asymptomatic human infection with HPAI H5N8 clade 2.3.4.4b viruses in poultry farm workers, linked to a poultry outbreak (9). However, a serologic study revealed the presence of antibodies to an HPAI H5N8 clade 2.3.4.4 virus in 61 of 760 serum samples from persons who had had contact with infected or deceased birds during the 2016–17 HPAI outbreaks in Russia (10), providing evidence of overlooked human infection.

The persistent circulation of clade 2.3.4.4 viruses among bird populations enables continuous reassortment with prevailing low pathogenicity avian influenza (LPAI) viruses. Together with intercontinental

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DOI: <https://doi.org/10.3201/eid2710.210297>

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dissemination through wild aquatic bird migration and potential interspecies transmission leading to mammalian adaptation, this circulation poses a major risk to human health should clade 2.3.4.4 viruses gain efficient human-to-human transmissibility, especially when immunity in the general population is lacking (1,2,8). Some HPAI H5N6/H5N8 clade 2.3.4.4 viruses have been shown to bind both α 2,6- (human) and α 2,3- (avian) linked sialic acid receptors, and more than half of HPAI H5N6 clade 2.3.4.4 isolates from humans found in GISAID acquired E627K or D701N substitutions in the polymerase basic (PB) 2 protein, which play prominent roles in mammalian adaptation of avian influenza viruses (8,11–13). An HPAI H5N8 clade 2.3.4.4 virus quickly acquired virulence markers, enhancing its virulence in mice and replication and polymerase activity in human cell lines within 5 murine passages (14), suggesting potential rapid adaptation after repeated virus introduction.

Surveillance efforts and characterization of clade 2.3.4.4 viruses provide insight into their pathogenicity and transmissibility, which can be used to prevent future outbreaks and assess zoonotic potential. Experimentally inoculated ferrets, mice, and guinea pigs displayed considerable variation in pathogenicity, and transmission by direct contact was demonstrated in guinea pig and ferret models (8,11–13,15,16). A few studies have also indicated efficient replication of HPAI H5N6 clade 2.3.4.4 viruses in human bronchus and lung explants and in primary human bronchial epithelial cells, which might have been linked to their respective successful infection of humans and direct-contact transmission among ferrets (16,17).

To further build on these findings, we used physiologically relevant 3-dimensional human airway organoids and primary human alveolar epithelial cells to investigate the tropism and innate host responses of 5 HPAI H5N6/H5N8 clades 2.3.4.4b, e, and h avian isolates from 2016–2018. We compared these responses to those of earlier human isolates of HPAI H5N1 clades 0 and 2.3.2.1b, HPAI H5N6 clade 2.3.4.4, an influenza A(H1N1)pdm09 virus (pH1N1), and an LPAI H5N8 virus.

Methods

Viruses

We used 3 HPAI H5N6 avian isolates: A/environment/Hong Kong/WCRB-01/2018 (avHPAI H5N6/DK01) of clade 2.3.4.4h, isolated from the outside of a chilled duck (GISAID accession no. EPI_ISL_885144); A/spoonbill/HK/17-18259/2017

(avHPAI H5N6/18259) of clade 2.3.4.4b, isolated from a trachea tissue sample of a dead black-faced spoonbill (GISAID accession no. EPI_ISL_885145); and A/northern pintail/HK/MP692/2016 (avHPAI H5N6/MP692) of clade 2.3.4.4e, isolated from a fecal sample of a northern pintail (GISAID accession no. EPI_ISL_885147). We also used 2 HPAI H5N8 clade 2.3.4.4b avian isolates: A/chicken/Egypt/F1366A/2017 (avHPAI H5N8/636099) (GISAID accession no. EPI_ISL_885148) and A/grey-headed gull/Uganda/200144/2017 (avHPAI H5N8/642613) (GISAID accession no. EPI_ISL_885149); 1 HPAI H5N6 clade 2.3.4.4 human isolate A/Guangzhou/39715/2014 (HPAI H5N6/39715) from the throat swab of a 59-year-old male patient on day 8 of illness (GenBank accession no. KP765785-KP765792); 2 HPAI H5N1 human isolates, A/Hong Kong/483/1997 (HPAI H5N1/483) of clade 0 isolated from a person with a fatal case (GenBank accession nos. GU052096-GU052104, AF258820, AF084277) and A/Shenzhen/1/2011 (HPAI H5N1/SZ1) of clade 2.3.2.1b (GISAID accession no. EPI_ISL_891209); LPAI H5N8 avian isolate A/northern pintail/Hong Kong/MP5883/2004 (avLPAI H5N8/MP5883) (GISAID accession no. EPI_ISL_885151); and pH1N1 virus A/Hong Kong/415742/2009.

We prepared virus stocks in MDCK cells with limited passages. To determine virus titers, we used 50% tissue culture infectious dose (TCID₅₀) assays.

Human Airway Organoids

We cultured human airway organoids from cells isolated from human lung tissue and infected in 6 log TCID₅₀/mL virus for 1 h at 37°C as previously described (18,19). We collected supernatant at 1, 24, 48, and 72 h after infection for virus titration by TCID₅₀ assay. We fixed organoids in 4% paraformaldehyde at postinfection hours 24 and 48 for immunohistochemical double staining and collected cell lysates at postinfection hour 24 for measurement of mRNA expression.

Primary Human Alveolar Epithelial Cells

We isolated alveolar epithelial cells from human lung tissues, cultured, and infected at multiplicities of infection (MOIs) of 0.01 and 2 for 1 h at 37°C as previously described (17,18). We collected supernatant at 1, 24, 48, and 72 h after infection for virus titration by TCID₅₀ assay and collected cell lysates at 24 h after infection to measure mRNA expression. We compiled a description of our detailed study methods (Appendix 1, <https://wwwnc.cdc.gov/EID/article/27/10/21-0297-App1.pdf>).

Results

Productive Replication

All 5 HPAI (H5N6/H5N8) avian isolates demonstrated productive replication in human airway

organoids and alveolar epithelial cells (MOI 0.01); by 72 h after infection, mean peak titers were 3.7–5.1 log TCID₅₀/mL for human airway organoids and 4.6–7.0 log TCID₅₀/mL for alveolar epithelial cells (Figure, panels A, B). Mean peak titers of HPAI H5 isolates

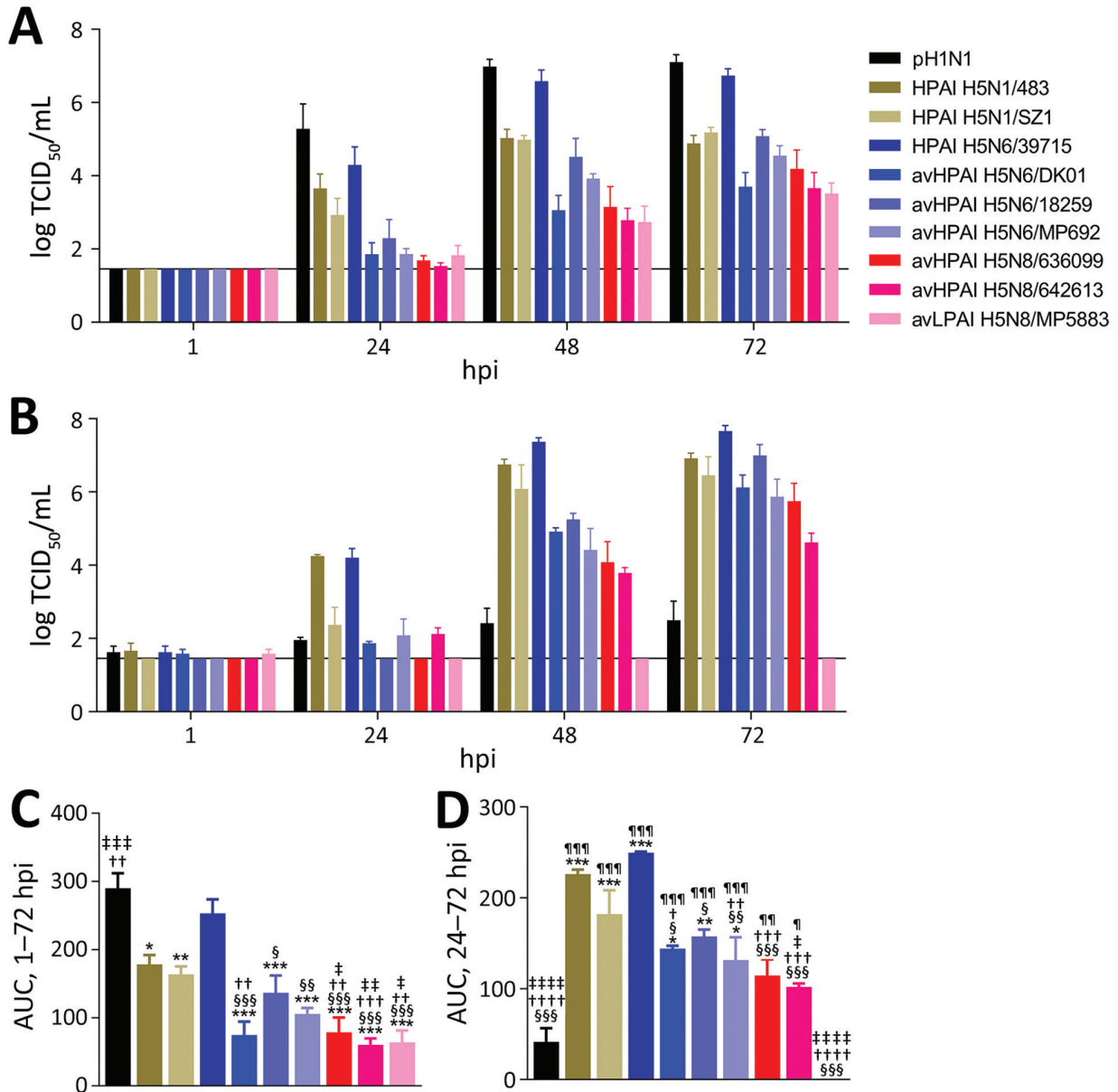


Figure. Replication kinetics of influenza A viruses. A, B) Replication in human airway organoids infected with 6 log TCID₅₀/mL virus (A) and primary human alveolar epithelial cells infected at multiplicity of infection 0.01 at 37°C (B). Virus titers in culture medium (mean ± SEM, n≥3) were determined by TCID₅₀ assays with a detection limit of 1.5 log TCID₅₀/mL, denoted by a solid line. Statistical significance between virus titers at each time point after infection is provided in Appendix 1 Figure 1 (<https://wwwnc.cdc.gov/EID/article/27/10/21-0297-App1.pdf>). C, D) The areas under the replication kinetic curves above the detection limit in human airway organoids from 1 to 72 hpi (C) and alveolar epithelial cells from 24 to 72 hpi (mean ± SEM, n ≥3) (D). Statistical significance between AUC values was analyzed by using 1-way analysis of variance with Bonferroni posttests. *p≤0.01; **p≤0.001; ***p≤0.0001 (compared with pH1N1); †p≤0.05; ††p≤0.01; †††p≤0.001; ††††p≤0.0001 (compared with HPAI H5N1/483); ‡p≤0.05; ‡‡p≤0.01; ‡‡‡p≤0.001; ‡‡‡‡p≤0.0001 (compared with HPAI H5N1/SZ1); §p≤0.01; §§p≤0.001; §§§p≤0.0001 (compared with HPAI H5N6/39715); ¶p≤0.01; ¶¶p≤0.001; ¶¶¶p≤0.0001 (compared with avLPAI H5N8/MP5883). AUC, area under the curve; av, avian; HPAI, highly pathogenic avian influenza; hpi, hours postinfection; LPAI, low pathogenicity avian influenza; pH1N1, influenza A(H1N1)pdm09 virus; TCID₅₀, 50% tissue culture infectious dose.

from humans were 5.0–6.7 log TCID₅₀/mL for human airway organoids and 6.5–7.7 log TCID₅₀/mL for alveolar epithelial cells. As expected, pH1N1 replicated to the highest mean peak titer of 7.1 log TCID₅₀/mL in human airway organoids but reached only 2.5 log TCID₅₀/mL in alveolar epithelial cells. AvLP AI H5N8/MP5883 replicated to a mean peak titer of 3.5 log TCID₅₀/mL in human airway organoids but did not show any detectable replication in alveolar epithelial cells. When we compared the replication kinetic areas under the curve (AUCs), which estimated the total quantity of virus released, we found comparable AUC values among the HPAI H5N6/H5N8 avian isolates but lower AUC values for HPAI H5N6/H5N8 avian isolates than for the HPAI H5N6/39715 human isolate in human airway organoids and in alveolar epithelial cells (Figure, panels C, D). The AUC values of HPAI H5N6/H5N8 avian isolates were also lower than those of pH1N1 in human airway organoids and the HPAI H5N1/483 human isolate in alveolar epithelial cells, except for the statistically insignificant AUC values between avHPAI H5N6/18259 and HPAI H5N1/483 in alveolar epithelial cells.

Cellular Tropism

According to immunohistochemistry double staining, avHPAI H5N6/18259, avHPAI H5N6/MP692, and avHPAI H5N8/636099 infected acetyl- α -tubulin-positive ciliated cells, SCGB1A1-positive/CC10-positive secretory club cells, MUC5AC-positive secretory goblet cells, and p63- α -positive basal cells, similar to pH1N1, HPAI H5N1/483, HPAI H5N6/39715, and avLP AI H5N8/MP5883 (Appendix 2 Figure 1, <https://wwwnc.cdc.gov/EID/article/27/10/21-0297-App2.pdf>). avHPAI H5N6/DK01 and HPAI H5N1/SZ1 infected ciliated cells, club cells, and goblet cells. avHPAI H5N8/642613 infected only club cells.

Proinflammatory Cytokine and Chemokine Induction

We studied induction of proinflammatory cytokines and chemokines by using HPAI H5N1/483 as a high inducing virus control and pH1N1 as a low inducing virus control. At 24 h after infection, HPAI H5N1/483 tended to induce higher mRNA levels of IFN- β , IFN- λ 1, CCL5, CXCL10, TNF α , IL-6, ISG15, and MX1 than most HPAI H5N6/H5N8 avian isolates in human airway organoids and in alveolar epithelial cells (MOI 2); we observed statistical significance for IFN- λ 1, CXCL10, and MX1 in human airway organoids and IFN- β , IFN- λ 1, CCL5, CXCL10, ISG15, and MX1 in alveolar epithelial cells (Appendix 2 Figure 2). We found no statistically significant differences in the

mRNA levels of these genes between HPAI H5N6/H5N8 avian isolates, HPAI H5N6/39715, pH1N1, and avLP AI H5N8/MP5883 in human airway organoids. Similarly, we detected only a few differences between their IFN- β , TNF α , ISG15, and MX1 mRNA levels in alveolar epithelial cells.

Molecular Comparisons

Molecular analysis revealed that all 5 HPAI H5N6/H5N8 avian isolates and the 3 HPAI H5 human isolates contained a 5 to 6 basic residue polybasic cleavage site (Appendix 2 Table), characteristic of a highly pathogenic phenotype. We detected amino acid differences in the receptor-binding sites and glycosylation sites of the hemagglutinin (HA) proteins, which may contribute to altered receptor-binding preference among the 9 HPAI/LPAI H5 viruses. Of note, all H5 viruses possessed ≥ 1 of the 7 observed HA amino acid mutations previously reported to increase virus binding to α 2,6-linked sialic acid receptors: 94N, 133A, 154D, 155N, 156A, 188I, and 189R (20–23). HPAI H5N6/H5N8 viruses and HPAI H5N1/SZ1 also contained a single or double HA amino acid mutation(s), 218Q and 223R, required for binding fucosylated α 2,3-linked sialic acid receptors (24). Of the 29 molecular marker positions associated with virulence, transmission, replication efficiency, and adaptation in mammals, which were differentially expressed among the 9 H5 viruses in neuraminidase (NA), PB2, PB1, PB1-F2, polymerase acidic, nucleocapsid, matrix (M) 1, M2, nonstructural (NS) 1 and NS2 proteins (11,17,25–34), most (20) molecular markers were found in HPAI H5N1/483 viruses; 8–11 in HPAI H5N1/SZ1 and HPAI H5N6 viruses, except for avHPAI H5N6/18259, which contained only 2, and 4–6 in HPAI/LPAI H5N8 viruses. The well-known mammalian adaptation marker PB2 627K was detected in only 2 HPAI H5 human isolates, HPAI H5N1/483 and HPAI H5N6/39715; PB2 701N was not detected in any of the H5 viruses. The NA 96A and M2 31N mutations, which confer resistance, were observed in avHPAI H5N6/18259 (resistant to zanamivir and oseltamivir) and avHPAI H5N6/MP692 (resistant to amantadine and rimantadine) (35, 36).

Receptor Binding

We used untreated and desialylated 0.5% turkey red blood cells (TRBCs) to determine the receptor-binding specificities of the HPAI H5N6/H5N8 avian isolates. Cleavage of α 2,3-linked unbranched sialic acid by Glyko Sialidase S (Agilent, <https://www.agilent.com>) reduced the hemagglutination of avHPAI H5N6/18259 by 16-fold but did not affect

hemagglutination of avHPAI H5N6/DK01, avHPAI H5N6/MP692, avHPAI H5N8/636099, and avHPAI H5N8/642613 (Table). On the other hand, cleavage of both α 2,3- and α 2,6-linked unbranched sialic acid by Glyko Sialidase C (Agilent) resulted in 2- to 16-fold drops in hemagglutination of avHPAI H5N6/DK01, avHPAI H5N6/MP692, avHPAI H5N8/636099, and avHPAI H5N8/642613. However, there was no further reduction in the level of hemagglutination of avHPAI H5N6/18259 in Sialidase C-treated versus Sialidase S-treated TRBCs. These data suggest that avHPAI H5N6/18259 predominantly binds α 2,3-linked sialic acid, but the other 4 HPAI H5N6/H5N8 avian isolates can bind to receptors with α 2,6 linkage. Treatment of TRBCs with Sialidase S and Sialidase C similarly abolished the hemagglutination of predominantly α 2,3-linked sialic acid binders HPAI H5N1/483 and HPAI H5N6/39715 (17, 18) and partially prevented the hemagglutination of HPAI H5N1/SZ1 and avLPAI H5N8/MP5883. The hemagglutination of predominantly α 2,6-linked sialic acid binder pH1N1 (37) was not affected by Sialidase S but was partly inhibited by Sialidase C. The ability of the tested viruses, apart from HPAI H5N1/483 and HPAI H5N6/39715, to maintain some hemagglutination to Sialidase C-treated TRBCs suggests their potential to bind to receptors other than sialic acid with α 2,3 and α 2,6 unbranched linkages.

Discussion

We demonstrated that the 5 HPAI H5N6/H5N8 clade 2.3.4.4b, e, and h avian isolates from 2016–2018 replicated productively and to similar competence in human airway organoids and alveolar epithelial cells. Replication was less efficient than that of pH1N1 and HPAI H5N6/39715 in human airway organoids and HPAI H5N1/483 and HPAI H5N6/39715 in alveolar epithelial cells. The isolates showed differential cellular tropism in human airway organoids; some infected all 4 major epithelial cell types, including ciliated

cells, club cells, goblet cells, and basal cells, similar to pH1N1 and the HPAI H5 human isolates. Compared with HPAI H5N1/483, HPAI H5N6/H5N8 clade 2.3.4.4 viruses induced fewer proinflammatory cytokines and chemokines.

Tropism of influenza virus for the human conducting airways and lower lung epithelial cells is a useful parameter for assessing its zoonotic and pandemic threat in terms of receptor-binding capacity, and the World Health Organization has listed infection of human bronchus explants as a reliable risk-assessment platform (38). In addition, replication in the conducting airways has been suggested as a prerequisite for influenza virus acquisition of efficient human-to-human transmission (38, 39). We have previously demonstrated that tropism and replication competence of influenza viruses in human airway organoids mimicked those found in human bronchus explants (18, 19). However, human airway organoids are more readily available for studies because they can be easily rescued from long-term cryopreservation and expanded.

In this study, the productive but lower replication competence of HPAI H5N6/H5N8 clade 2.3.4.4b, e, and h avian isolates compared with that of pH1N1, HPAI H5N1/483, and HPAI H5N6/39715 in human airway organoids, alveolar epithelial cells, or both, suggests lower zoonotic potential and transmissibility of these avian isolates in humans. Knowing that pH1N1 was a successful pandemic virus that infected an estimated 20%–27% of the world population during the first year of circulation (40) and that HPAI subtypes H5N1 and H5N6 are able to cause zoonotic infection but lack efficient human-to-human transmission to cause a pandemic to date, we could extrapolate that the risk for human-to-human transmission of HPAI H5N6/H5N8 clade 2.3.4.4b, e, and h avian isolates in this study is low but that zoonotic infection caused by direct contact with infected poultry or contaminated environment is possible, as

Table. Effects of desialylation on influenza A virus hemagglutination of turkey red blood cells*

Virus	0.5% Turkey red blood cells		
	Untreated	Sialidase S†	Sialidase C†
Influenza A(H1N1)pdm09	64	64	8
HPAI H5N1/483	256	0	0
HPAI H5N1/SZ1	64	4	4
HPAI H5N6/39715	128	0	0
avHPAI H5N6/DK01	128	128	32
avHPAI H5N6/18259	64	4	4
avHPAI H5N6/MP692	64	64	32
avHPAI H5N8/636099	128	128	8
avHPAI H5N8/642613	256	256	64
avLPAI H5N8/MP5883	32	4	4

*Hemagglutination titers were calculated as the reciprocal of the highest dilution that gave hemagglutination. Experiments were performed in triplicate and led to identical results. Av, avian; HPAI, highly pathogenic avian influenza.

†Agilent (<https://www.agilent.com>).

in the case of HPAI H5N6/39715 and most previous human cases of HPAI H5N6/H5N8 clade 2.3.4.4 infection (7, 8). Perhaps it would be best to confirm the virus transmissibility in animal models, such as ferrets and guinea pigs. However, the inability of HPAI H5N6/39715 to transmit among ferrets via respiratory droplets (41) makes it highly doubtful that the HPAI H5N6/H5N8 clade 2.3.4.4b, e, and h avian isolates in this study would transmit better. It has been shown that replication in human bronchus explants correlates with respiratory droplet transmission of swine influenza viruses in ferrets (39). Respiratory droplet transmission of HPAI H5N6/H5N8 clade 2.3.4.4 viruses has not been demonstrated in any animal model examined (8,12,13,16,41,42), which is consistent with their lack of human-to-human spread. However, direct contact transmission of some HPAI H5N6 clade 2.3.4.4 viruses was previously demonstrated in ferrets and guinea pigs (8,12,13,16), thus indicating potential risk.

Although transmissibility of the HPAI H5N6/H5N8 clade 2.3.4.4 viruses in humans seems to be low, persons with frequent exposure to poultry and wild birds should be educated to practice precautionary measures because of ongoing outbreaks among avian species. Repeated virus introductions provide a chance for adaptation to enhance virus replication efficiency, virulence, and transmissibility in humans (14-43). An avian influenza HPAI H5N1 virus became airborne transmissible among ferrets after acquiring mutations during passage in ferrets (43). Moreover, reassortments of viral gene segments in avian species are continuously associated with the evolution of H5Nx viruses (1,2,8).

The lower replication competence of avHPAI H5N6/18259, avHPAI H5N6/MP692, and avHPAI H5N8/636099 compared with that of HPAI H5N6/39715 in human airway organoids, despite being similarly able to infect all 4 major epithelial cell types, may partially be explained by their lack of the mammalian adaptation marker PB2 627K that is present in HPAI H5N6/39715. PB2 627K enhances polymerase activity and avian virus replication in mammalian cells. Likewise, the presence of PB2 627E instead of 627K and a higher preference for α 2,6-linked sialic acid among the 5 HPAI H5N6/H5N8 avian isolates may partially be accounted for by their lower replication competence compared with that of HPAI H5N1/483 and HPAI H5N6/39715 in alveolar epithelial cells, which are predominantly lined with α 2,3-linked sialic acid receptors (44).

Because human respiratory epithelium is the primary target for influenza viruses, knowledge of

the innate host responses in the respiratory epithelium, especially that of the alveolar epithelium, on severe influenza infection, such as infection with HPAI H5N1, helps elucidate the pathogenesis of the dysregulated cytokine responses and severe pneumonia associated with the disease. In this study, HPAI H5N6/H5N8 clade 2.3.4.4 viruses, including the HPAI H5N6/39715 human isolate, were found to be low inducers of proinflammatory cytokines and chemokines in alveolar epithelial cells and in human airway organoids, which is unlikely to contribute to the pathogenesis of severe disease. In connection, we found fewer molecular markers associated with virulence in the tested clade 2.3.4.4 viruses compared with HPAI H5N1/483. Our data are consistent with those from previous studies that reported low cytokine/chemokine induction of HPAI H5N6 clade 2.3.4.4 viruses in alveolar epithelial cells and low virulence of clade 2.3.4.4 HPAI H5N8 viruses in humans, ferrets, and mice (9,10,16,17,42,45,46). However, those findings cannot help explain the high fatality rate of HPAI H5N6 clade 2.3.4.4 infection in humans and the high cytokine/chemokine levels in the serum of some patients infected with HPAI H5N6 clade 2.3.4.4, which are comparable to those infected with HPAI H5N1 (7). One possible explanation may be the differences in virus strains involved. Some studies have pointed out that HPAI H5N6 clade 2.3.4.4 viruses exhibited pronounced strain-specific heterogeneity regarding their capacity to cause severe and fatal disease (11,13). Another reason maybe the differences in the patients' health conditions, including their ages and underlying medical conditions, in naturally infected patients (47). Moreover, apart from innate host responses in the epithelium, other factors (e.g., responses from immune cells and viral tropism for deeper body cells/tissues) are likely to contribute to the final outcomes of the viral infection.

Previous studies that used different receptor-binding assays demonstrated both dual receptor-binding preference and preference to α 2,3-linked sialic acid of H5 clade 2.3.4.4 viruses (13,16,17,48). Although avHPAI H5N6/18259 predominantly bound α 2,3-linked sialic acid in our desialylation-hemagglutination assay, it showed comparable productive replication to the 4 α 2,6-linked sialic acid-binding HPAI H5N6/H5N8 avian isolates in human airway organoids, which are predominantly lined with α 2,6-linked sialic acid receptors (19). It also showed tropism for all 4 major epithelial cell types infected by the predominantly α 2,6-linked sialic acid binder pH1N1 in human airway organoids.

Likewise, Kwon et al. showed efficient replication of 2 HPAI H5N6/H5N8 clade 2.3.4.4 avian viruses with strong preferential binding to α 2,3-linked sialic acid in human bronchial epithelial cells (16). These data suggest that the α 2,3/ α 2,6 linkage type alone may not be sufficient to successfully determine influenza virus tropism in the human respiratory tract. More comprehensive but focused glycan arrays that express the wide spectrum of relevant glycans in human airways are needed (37). Unfortunately, only a few glycans present in currently available glycan arrays are found in the human respiratory tract.

Because our desialylation-hemagglutination assay involved sialidases that cleaved only unbranched α 2,3- and α 2,6-linked sialic acid, binding of the H5 viruses to branched sialic acid could not be assessed, which might have contributed to the maintenance of hemagglutination titers in Sialidase S-treated and Sialidase C-treated TRBCs. Fucosylated α 2,3-linked sialic acid is a potential branched receptor. It was shown to bind H5 HA clade 2.3.4.4 proteins with substitutions in K218Q, S223R, or both, which can be found in our tested HPAI H5N6/H5N8 clade 2.3.4.4 viruses (24). Complex glycans containing fucosylated structures with sialylation, including sialyl-Lewis X (NeuAc α 2-3Gal β 1-4[Fuca1-3]GlcNAc) and sialyl-Lewis A (NeuAc α 2-3Gal β 1-3[Fuca1-4]GlcNAc), have been detected in human lung tissues and the epithelium of small airways (49). Binding to these glycans might increase the infectivity and hence replication of clade 2.3.4.4 viruses in human airways. In contrast, earlier HPAI H5N1 viruses without the double HA amino acid substitutions have reduced binding to glycans that are fucosylated at the penultimate GlcNAc residue (24,50).

In conclusion, the productive viral replication and tropism for different cell types in human airway organoids and alveolar epithelial cells suggest zoonotic potential of the 5 HPAI H5N6/H5N8 clade 2.3.4.4b, e, and h avian isolates from 2016–2018. However, the risk for human-to-human transmission seems to be low. The low levels of proinflammatory cytokines and chemokines induced by these viruses in the human respiratory epithelium are unlikely to contribute to the pathogenesis of severe disease. However, the persistent circulation of clade 2.3.4.4 viruses among avian populations and periodic infection of human hosts enable ongoing evolution of the viruses with the possibility of acquiring better transmissibility, higher pathogenicity, or both, in humans. Therefore, education, vaccine development, surveillance, and risk assessment surrounding these viruses should continue.

Acknowledgments

We thank Kevin Fung for assistance with immunohistochemistry and Hung Sing Li, Rachel H.H. Ching, Amy W.Y. Tang, Samuel M.S. Chan, and Joe K.C. Tsun for technical support.

This work was supported by Theme Based Research Scheme (T11-705/14N, T11-712/19-N), Research Grants Council, Hong Kong SAR, China; and the US National Institute of Allergy and Infectious Diseases under Centers of Excellence for Influenza Research and Surveillance (HHSN272201400006C).

C.H.T.B. and D.I.T.K. designed and coordinated the study, planned and conducted the experiments, and analyzed the results. C.H.T.B. wrote the manuscript. H.W.Y., K.-C.N., and D.K.W.C. conducted the experiments and analyzed the results. R.J.W. provided HPAI H5N8 viruses. J.M.N., J.S.M.P., and K.P.Y.H. designed the study and reviewed the manuscript. M.C.W.C. did the overall coordination and design of the study, analyzed the results, and reviewed the manuscript.

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Risk Assessment for Highly Pathogenic Avian Influenza A(H5N6/H5N8) Clade 2.3.4.4 Viruses

Appendix 1

Methods

50% Tissue Culture Infectious Dose (TCID₅₀) assay

Confluent 96-well MDCK cell plates were seeded one day before. Cells were then washed with 1x phosphate buffered saline (PBS), pH 7.4 without Ca²⁺ or Mg²⁺, and filled with new medium: MEM (Gibco) supplemented with 100 U/ml Penicillin-Streptomycin (Gibco), 0.025 M HEPES (Gibco), and 1 µg/ml TPKC-treated trypsin from bovine pancreas (Sigma-Aldrich). Virus was serially diluted at half-log₁₀ dilution and added to the cell plates in quadruplicates. Plates were incubated in a humidified incubator at 37°C and 5% CO₂ until 72 hours post infection (hpi). Karber method was used to determine the viral dilution endpoints leading to cytopathic effect (CPE) in 50% of inoculated wells.

Human lung tissues

Human lung tissues were removed from patients undergoing surgical resection as part of a routine clinical care in Hong Kong. After fulfilling all diagnostic requirements, fresh non-tumour residual lung tissue was used for this study. Informed consent was obtained from patients and approval was granted by the Institutional Review Board of the University of Hong Kong and the Hospital Authority (Hong Kong West) (approval no: UW 18-196).

Human airway organoids – Isolation and Culture

Human lung tissues were minced and washed with AdDF⁺⁺⁺ basic medium, which consisted of Advanced DMEM/F-12 (Gibco) supplemented with 1x GlutaMAX[™] (Gibco), 10 mM HEPES (Gibco), and 100 U/ml Penicillin-Streptomycin (Gibco). Tissues were digested with 2 mg/ml collagenase type II (Sigma-Aldrich) in a 37°C shaker for 1 h. 2% fetal bovine serum (FBS) was added to stop the digestion. The tissue suspension was added with 100 µg/ml DNase I

(Sigma-Aldrich), sheared with plastic pipettes, and strained with 100 μm and 70 μm filters. The collected filtrate was centrifuged at $600 \times g$ for 5 min at 4°C . Supernatant was removed. The cell pellet was resuspended in red blood cell lysis buffer (Roche) and incubated at room temperature for 5 min, followed by the addition of AdDF+++ basic medium and centrifugation at $600 \times g$ for 5 min at 4°C . 10 mg/ml cold Cultrex growth factor reduced basement membrane extract type 2 Matrigel (Trevigen) was added to the cell pellet and 40 μl droplets of the cell-in-Matrigel suspension was aliquoted to prewarmed 24-well suspension culture plates. The plates were incubated at 37°C for 15-30 min. 1 ml organoid medium was added to each well as previously described (1,2). The plates were incubated in a humidified incubator at 37°C and 5% CO_2 . Medium was changed every 4 days and organoids were passaged every 2 weeks. To passage, organoids-in-Matrigel droplets were resuspended in cold AdDF+++ , sheared with flamed glass Pasteur pipettes, and centrifuged at $400 \times g$ for 5 min at 4°C . Organoid fragments were re-embedded in new cold Matrigel with the usual expansion of 6 times the original Matrigel droplet number. After 14 days in culture at 37°C and 5% CO_2 , human airway organoids were ready for infection.

Human airway organoids – Infection

Human airway organoids of approximately 100 μm in diameter were removed from Matrigel droplets and slightly broken open by shearing with flamed glass Pasteur pipettes. Around 100–200 organoids were infected with each virus at 6 log $\text{TCID}_{50}/\text{ml}$ for 1 h at 37°C . Organoids were washed 3 times: twice with AdDF+++ basic medium and once with organoid medium. They were re-embedded in Matrigel and cultured in organoid medium in a humidified incubator at 37°C and 5% CO_2 . Culture supernatant was collected at 1, 24, 48 and 72 hpi for virus titration by TCID_{50} assay. Organoids were fixed in 4% paraformaldehyde at 24 and 48 hpi for immunohistochemical double staining. Organoid lysates were collected at 24 hpi by adding Buffer RLT (Qiagen) supplemented with 1% β -mercaptoethanol. Experiments were performed with organoids isolated from at least 4 different donors.

Primary human alveolar epithelial cells – Isolation and Culture

Human lung tissues were minced and washed 3 times using HBSS (Gibco) supplemented with 0.7 mM sodium bicarbonate (Gibco) at pH 7.4. Tissues were digested using 0.5% trypsin (Gibco) and 4 U/ml elastase (Worthington Biochemical Corporation) in a 37°C water bath for 40 min. DMEM/F12 medium (Gibco) supplemented with 40% FBS and 350 U/ml DNase I

(Sigma-Aldrich) were added to stop the digestion. The tissue suspension was pipetted up and down with 10 ml plastic pipettes for 10 min and strained through 50 µm filters. The filtrate was centrifuged at 1500 rpm for 5 min. Supernatant was removed and the cell pellet was resuspended in a 1:1 mixture of DMEM/F12 medium and small airway growth medium (SAGM) (Lonza) supplemented with 5% FBS and 350 U/ml DNase I. The cell suspension was seeded to tissue culture flasks (Corning) and incubated in a humidified incubator at 37°C and 5% CO₂ for 90 min. Cells not adhered to the flasks were collected and centrifuged at 1500 rpm for 5 min. Supernatant was removed. The cell pellet resuspended in SAGM supplemented with 100 U/ml Penicillin-Streptomycin (Gibco) was seeded to new tissue culture flasks. Cells were kept in a humidified incubator at 37°C and 5% CO₂. After 60 h, culture medium was changed every day until reaching 75% confluence. Cells were then trypsinized and seeded for infection.

Primary human alveolar epithelial cells – Infection

1 x 10⁵ cells/well were seeded in 24-well tissue culture plates a day before. Attached cells were washed 3 times with 1x PBS, pH 7.4 without Ca²⁺ or Mg²⁺, and infected at multiplicities of infection (MOIs) of 0.01 and 2 for 1 h at 37°C and 5% CO₂. The infected cells were washed 3 times with 1x PBS and replenished with 1 ml SAGM (Lonza) supplemented with 100 U/ml Penicillin-Streptomycin (Gibco) and 0.25 µg/ml TPCK-treated trypsin from bovine pancreas (Sigma-Aldrich). They were cultured in a humidified incubator at 37°C and 5% CO₂. Culture supernatant was collected at 1, 24, 48, and 72 hpi for virus titration by TCID₅₀ assay. Cell lysates were collected at 24 hpi by adding 350 µl Buffer RLT (Qiagen) with 3.5 µl β-mercaptoethanol to each well. Experiments were performed with cells isolated from 3 different donors.

Immunohistochemistry double staining

The staining procedures were as previously described with slight modifications (1,2). Infected organoids were washed with cold AdDF+++ and removed from Matrigel droplets prior to being fixed in 4% paraformaldehyde. Fixed tissues were embedded in paraffin. After sectioning and deparaffinization, the sections were incubated with 0.05% Pronase (Roche) at 37°C for 16 min and 3% H₂O₂ at room temperature for 10 min. Subsequently, ImmPRESS® HRP Anti-Mouse IgG (Peroxidase) Polymer Detection Kit, made in Horse (Vector Laboratories) were used for staining with the first antibody according to the manufacturer's instructions. Briefly, sections were blocked with 2.5% horse serum. Anti-influenza A virus (IAV) nucleoprotein-specific mouse monoclonal antibody HB65 (European Veterinary Laboratory) was

added to the sections at a dilution of 1/10 and incubated at room temperature for 1 h. After a brief wash, the ImmPRESS Reagent was added to the sections and incubated for 1 h at room temperature. Sections were then rinsed, and the slides were developed with 0.5 mg/ml DAB (Vector Laboratories) for 2-3 min or VectorRed (Vector Laboratories) for 10 min. For staining with the second antibody, the sections were first microwaved at 95°C for 15 min in 10 mM citrate buffer, pH 6.0. ImmPRESS®-AP Anti-Mouse IgG (alkaline phosphatase) Polymer Detection Kit (Vector Laboratories) was used with 1/1000 diluted anti-acetylated α tubulin (Santa Cruz) antibody and 1/50 diluted anti-MUC5AC (ThermoFisher) antibody while ImmPRESS®-AP Anti-Rabbit IgG (alkaline phosphatase) Polymer Detection Kit (Vector Laboratories) was used with 1/100 diluted anti-SCGB1A1/CC10 (Protein-tech) antibody and 1/100 diluted anti-p63- α (Cell Signaling Technology) antibody according to the manufacturer's instructions. Incubation with the cell-type specific marker antibodies and the ImmPRESS Reagents took 60-90 min and 60 min, respectively, at room temperature. The sections were developed using VectorRed (Vector Laboratories) or DAB for 3 min. The nuclei were counterstained with Mayer's Haematoxylin for 10 seconds and the sections were blued with Scott's tap water, air dried, and mounted with Permount (Fisher Scientific).

Cytokine and chemokine expression

RNA was extracted from cell lysates using MiniBEST universal RNA extraction kit (Takara) and reverse transcribed using PrimerScript RT reagent Kit (Takara). mRNA expression was quantified using real-time PCR amplification with SYBR *Premix Ex Taq II* (Tli RNase H Plus) (Takara) and an ABI ViiA™ 7 real-time PCR system (Applied Biosystems). The procedures were performed according to the manufacturers' instructions. Absolute mRNA copy numbers of β -actin (forward primer: 5'-TGGATCAGCAAGCAGGAGTATG-3' and reverse primer: 5'-GCATTTGCGGTGGACGAT-3'), IAV matrix (M) gene (forward primer: 5'-GGCATTTTGGACAAAKCGTCTA-3' and reverse primer: 5'-CTTCTAACCGAGGTCGAAACG-3'), interferon- β (IFN β) (forward primer: 5'-CAACTTGCTTGGATTCTACAAAG-3' and reverse primer: 5'-TGCCACAGGAGCTTCTGACA-3'), interferon- λ 1 (IFN- λ 1) (forward primer: 5'-GCCCCAAAAGGAGTCCG-3' and reverse primer: 5'-AGGTTCCCATCGGCCACATA-3'), C-C motif chemokine ligand 5 (CCL5) (forward primer: 5'-CTTTGCCAGGGCTCTGTGA-3' and reverse primer: 5'-GCAGTGTTCCCTCCCCTCCTT-3'), C-X-C motif chemokine 10

(CXCL10) (forward primer: 5'-ATTATTCCTGCAAGCCAATTTTG-3' and reverse primer: 5'-TCACCCTTCTTTTTCATTGTAGCA-3'), tumor necrosis factor α (TNF α) (forward primer: 5'-GCAGGTCTACTTTGGGATCATTG-3' and reverse primer: 5'-GCGTTTGGGAAGGTTGGA-3'), interleukin 6 (IL-6) (forward primer: 5'-GCATGGGCACCTCAGATTGT-3' and reverse primer: 5'-TGCCCAGTGGACAGGTTTCT-3'), interferon-stimulated gene 15 (ISG15) (forward primer: 5'-CAAATGCGACGAACCTCTGA-3' and reverse primer: 5'-CCGCTCACTTGCTGCTTCA-3'), and interferon-induced GTP-binding protein Mx1 (MX1) (forward primer: 5'-GAGGCCAGCAAGCGCAT-3' and reverse primer: 5'-TGGAGCATGAAGAACTGGATGA-3') were determined with standard curve method (2,3). mRNA expression of all genes was normalized to that of β -actin.

Desialylation–haemagglutination assay

The effect of desialylation on IAV haemagglutination of Turkey red blood cells (TRBCs) was studied as previously described (2,3). TRBCs (Lampire) in Alsevers were washed and diluted to 0.5% in 1x PBS, pH 7.4 without Ca²⁺ or Mg²⁺. They were treated with either 20 mU/ml Glyko® Sialidase STM (Prozyme) or 200 mU/ml Glyko® Sialidase CTM (Aglilent) for 2 h at 37°C. After the treatment, TRBCs were washed with 1x PBS, centrifuged at 2000 rpm for 5 min, and resuspended in 1x PBS to 0.5%. Two-fold serial dilutions of viruses were carried out with 1x PBS in Greiner CELLSTAR® Clear V-bottom 96-well plates (Sigma-Aldrich) in triplicates. 50 μ l of the treated or untreated 0.5% TRBCs were added to 50 μ l of the diluted viruses/1x PBS (negative control) in each well. The plates were incubated at room temperature for 20 min. The haemagglutination titres were calculated as the reciprocal of the highest dilution that gave haemagglutination.

Phylogeny analysis

Clade classification was performed by comparative phylogenetic analysis. Seventy-one selected representative highly pathogenic avian influenza (HPAI) H5 HA sequences in Global Initiative on Sharing All Influenza Data (GISAID) and GenBank between 2007 and 2021 (4) (Appendix 1 Table) were used to build a phylogenetic tree based on the nucleotide sequences coding for the mature HA1 protein in this study. Multiple sequence alignment was performed using the MUSCLE algorithm. Phylogenetic analysis was inferred by using the maximum likelihood method and the Tamura-Nei model in MEGA version X. Stability of the phylogenetic tree branch topology was tested using 1000 bootstrap replicates.

Statistical and sequencing analysis

Area Under Curve (AUC) and statistical analyses were performed using GraphPad Prism, version 8.4.3. AUC values were calculated as the areas under the replication kinetic curves from 1 or 24 to 72 hpi above the TCID₅₀ assay detection limit (1.5 log TCID₅₀/ml) using the trapezoid rule (5). AUC values and mRNA levels were compared by one-way analysis of variance (one-way ANOVA) with *Bonferroni* post-tests. Viral titres at each time point post-infection were compared by two-way ANOVA with *Bonferroni* post-tests. Sequence alignment and analyses were conducted using MEGA version X.

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5. GraphPad Software. Area under the curve [cited 2021 Jun 7]. https://www.graphpad.com/guides/prism/latest/statistics/stat_area_under_the_curve.htm

Appendix 1Table. Highly pathogenic avian influenza (HPAI) H5 strains isolated between 2007 and 2021 and their HA sequence accession numbers in Global Initiative on Sharing All Influenza Data (GISAID) and GenBank

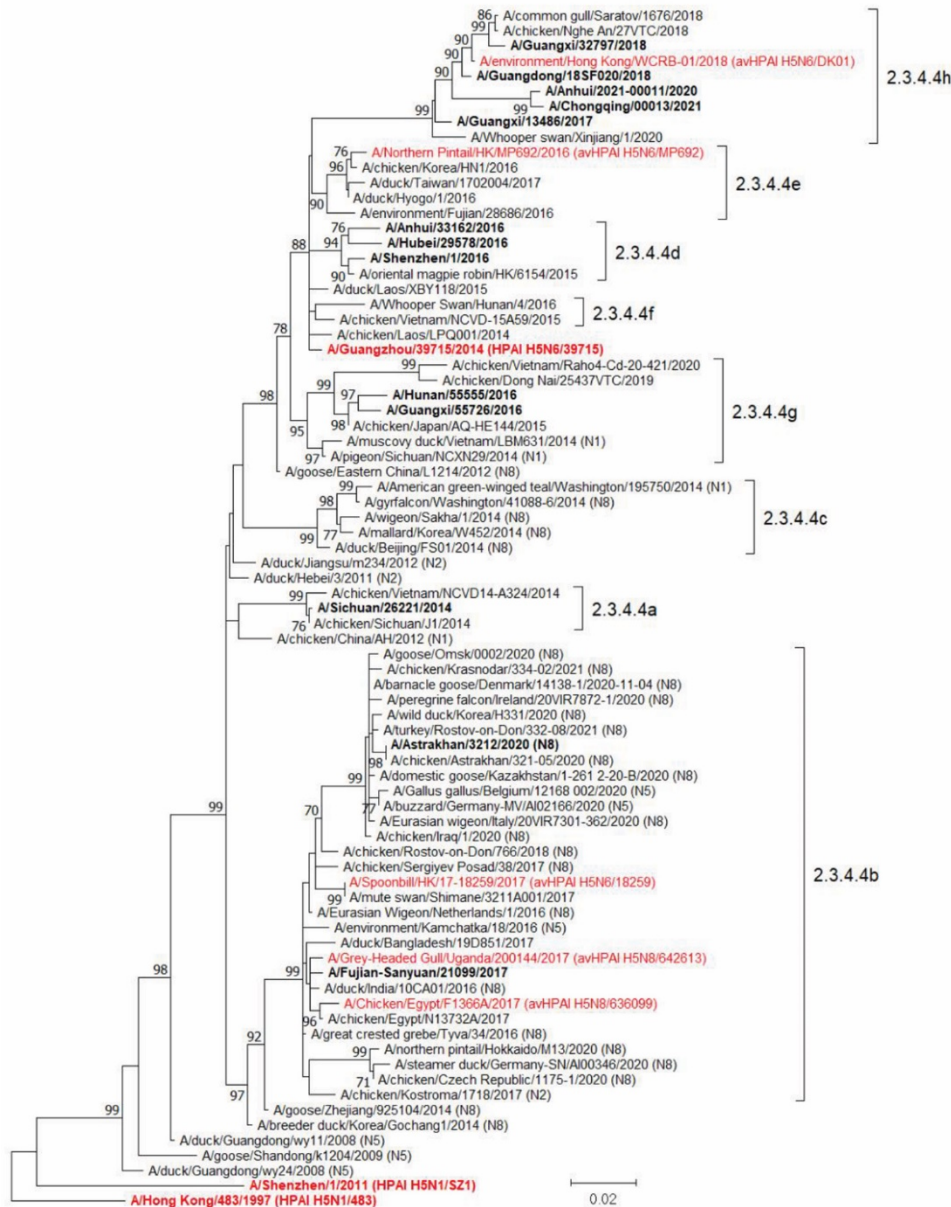
Clades	Reference strains*	Accession no.	
2.3.4.4a	A/chicken/Sichuan/J1/2014	EPI675770	
	A/Sichuan/26221/2014	EPI533583	
	A/chicken/Vietnam/NCVD14-A324/2014	EPI894943	
2.3.4.4b	A/wild duck/Korea/H331/2020 (N8)	EPI1846698	
	A/chicken/Astrakhan/321-05/2020 (N8)	EPI1846985	
	A/Astrakhan/3212/2020 (N8)	EPI1846961	
	A/turkey/Rostov-on-Don/332-08/2021 (N8)	EPI1848742	
	A/buzzard/Germany-MV/AI02166/2020 (N5)	EPI1811564	
	A/Gallus_gallus/Belgium/12168_002/2020 (N5)	EPI1814627	
	A/peregrine_falcon/Ireland/20VIR7872-1/2020 (N8)	EPI1841769	
	A/barnacle_goose/Denmark/14138-1/2020-11-04 (N8)	EPI1813753	
	A/chicken/Krasnodar/334-02/2021 (N8)	EPI1848790	
	A/domestic_goose/Kazakhstan/1-261_2-20-B/2020 (N8)	EPI1811592	
	A/goose/Omsk/0002/2020 (N8)	EPI1813121	
	A/Eurasian_wigeon/Italy/20VIR7301-362/2020 (N8)	EPI1843650	
	A/chicken/Iraq/1/2020 (N8)	EPI1811628	
	A/chicken/Rostov-on-Don/766/2018 (N8)	EPI1270931	
	A/chicken/Sergiyev Posad/38/2017 (N8)	EPI961459	
	A/mute swan/Shimane/3211A001/2017	EPI1127538	
	A/Eurasian_Wigeon/Netherlands/1/2016 (N8)	EPI1023576	
	A/chicken/Egypt/N13732A/2017	MH998393	
	A/duck/India/10CA01/2016 (N8)	EPI858836	
	A/Fujian-Sanyuan/21099/2017	EPI1202729	
	A/environment/Kamchatka/18/2016 (N5)	EPI961478	
	A/duck/Bangladesh/19D851/2017	EPI1329121	
	A/great crested grebe/Tyva/34/2016 (N8)	EPI823460	
	A/northern pintail/Hokkaido/M13/2020 (N8)	EPI1818402	
	A/chicken/Czech Republic/1175-1/2020 (N8)	EPI1667598	
	A/steamer duck/Germany-SN/AI00346/2020 (N8)	EPI1721676	
	A/chicken/Kostroma/1718/2017 (N2)	EPI1158108	
2.3.4.4c	A/gyrfalcon/Washington/41088-6/2014 (N8)	EPI569390	
	A/American green-winged teal/Washington/195750/2014 (N1)	EPI573286	
	A/wigeon/Sakha/1/2014 (N8)	EPI553349	
	A/mallard/Korea/W452/2014 (N8)	EPI542628	
2.3.4.4d	A/duck/Beijing/FS01/2014 (N8)	EPI543002	
	A/oriental magpie robin/HK/6154/2015	EPI1060783	
	A/Shenzhen/1/2016	EPI687704	
	A/Anhui/33162/2016	EPI1098355	
2.3.4.4e	A/Hubei/29578/2016	EPI961183	
	A/duck/Taiwan/1702004/2017	EPI915870	
	A/duck/Hyogo/1/2016	EPI866708	
	A/chicken/Korea/HN1/2016	EPI866085	
2.3.4.4f	A/environment/Fujian/28686/2016	EPI1333910	
	A/chicken/Vietnam/NCVD-15A59/2015	EPI895070	
2.3.4.4g	A/Whooper Swan/Hunan/4/2016	EPI1639951	
	A/chicken/Vietnam/Raho4-Cd-20-421/2020	EPI1853938	
	A/chicken/Dong Nai/25437/VTC/2019	EPI1665384	
	A/Hunan/55555/2016	EPI873668	
	A/Guangxi/55726/2016	EPI873669	
	A/chicken/Japan/AQ-HE144/2015	EPI946761	
	A/pigeon/Sichuan/NCXN29/2014 (N1)	EPI590898	
2.3.4.4h	A/muscovy duck/Vietnam/LBM631/2014 (N1)	AB979455	
	A/common gull/Saratov/1676/2018	EPI1355418	
	A/chicken/Nghe An/27VTC/2018	EPI1583716	
	A/Guangxi/32797/2018	EPI1352829	
	A/Guangdong/18SF020/2018	EPI1352813	
	A/Anhui/2021-00011/2020	EPI1848299	
	A/Chongqing/00013/2021	EPI1848291	
	A/Whooper swan/Xinjiang/1/2020	EPI1718942	
	A/Guangxi/13486/2017	EPI1352861	
	Others	A/chicken/China/AH/2012 (N1)	EPI499478
		A/chicken/Laos/LPQ001/2014	EPI550752
A/goose/Eastern China/L1214/2012 (N8)		EPI703597	
A/duck/Jiangsu/m234/2012 (N2)		EPI399960	
A/goose/Zhejiang/925104/2014 (N8)		EPI681300	
A/breeder duck/Korea/Gochang1/2014 (N8)		EPI509698	
A/duck/Hebei/3/2011 (N2)	EPI431456		

Clades	Reference strains*	Accession no.
	A/duck/Guangdong/wy11/2008 (N5)	EPI493817
	A/goose/Shandong/k1204/2009 (N5)	EPI442001
	A/duck/Guangdong/wy24/2008 (N5)	EPI493833
	A/duck/Laos/XBY118/2015	EPI1850997

*NA subtypes other than N6 are indicated in brackets.

		Reference virus										
		H1N1 pdm	HPAI H5N1/483	HPAI H5N1/SZ1	HPAI H5N6/39715	avHPAI H5N6/DK01	avHPAI H5N6/18259	avHPAI H5N6/MP692	avHPAI H5N8/636099	avHPAI H5N8/642613	avLPAI H5N8/MP5883	
24 hpi - human airway organoids												
Comparator virus	pH1N1											
	HPAI H5N1/483	> (*)										
	HPAI H5N1/SZ1	> (****) ns										
	HPAI H5N6/39715	ns	ns	ns								
	avHPAI H5N6/DK01	> (****) > (**)	ns	> (****)								
	avHPAI H5N6/18259	> (****) ns	ns	> (**)	ns							
	avHPAI H5N6/MP692	> (****) > (*)	ns	> (****) ns	ns							
	avHPAI H5N8/636099	> (****) > (**)	ns	> (****) ns	ns	ns						
	avHPAI H5N8/642613	> (****) > (**)	ns	> (****) ns	ns	ns	ns					
	avLPAI H5N8/MP5883	> (****) > (**)	ns	> (****) ns	ns	ns	ns	ns				
	48 hpi - human airway organoids											
	Comparator virus	pH1N1										
HPAI H5N1/483		> (**)										
HPAI H5N1/SZ1		> (****) ns										
HPAI H5N6/39715		ns	ns	< (*)								
avHPAI H5N6/DK01		> (****) > (**)	> (**)	> (****)								
avHPAI H5N6/18259		> (****) ns	ns	> (**)	ns							
avHPAI H5N6/MP692		> (****) ns	ns	> (****) ns	ns							
avHPAI H5N8/636099		> (****) > (**)	> (**)	> (****) ns	ns	ns						
avHPAI H5N8/642613		> (****) > (****)	> (****)	> (****) ns	> (*)	ns	ns					
avLPAI H5N8/MP5883		> (****) > (**)	> (**)	> (****) ns	> (*)	ns	ns	ns				
72 hpi - human airway organoids												
Comparator virus		pH1N1										
	HPAI H5N1/483	> (****)										
	HPAI H5N1/SZ1	> (**)	ns									
	HPAI H5N6/39715	ns	< (**)	ns								
	avHPAI H5N6/DK01	> (****) ns	ns	> (****)								
	avHPAI H5N6/18259	> (**)	ns	ns	ns							
	avHPAI H5N6/MP692	> (****) ns	ns	> (**)	ns	ns						
	avHPAI H5N8/636099	> (****) ns	ns	> (****) ns	ns	ns						
	avHPAI H5N8/642613	> (****) ns	> (*)	> (****) ns	ns	ns	ns					
	avLPAI H5N8/MP5883	> (****) ns	> (*)	> (****) ns	ns	ns	ns	ns				

Appendix 1 Figure 1. Statistical significance between viral titres at each time point post-infection in A) human airway organoids (Figure, panel A) and B) AECs (Figure, panel B) was calculated by two-way ANOVA with *Bonferroni* post-tests. No statistically significant differences were observed between the viral titres at 1 hour post infection (hpi). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$; >: reference virus value is higher than that of the comparator virus; <: reference virus value is lower than that of the comparator virus; ns: nonsignificant.



Appendix 1 Figure 2. Phylogenetic relationships of HA genes of A(H5) highly pathogenic avian influenza (HPAI) viruses. The nucleotide sequences coding for the mature HA1 protein were used for analysis. A maximum-likelihood tree using the A(H5) genes of the 8 HPAI viruses in this study (in red) and 71 representative A(H5) HA genes (4) (Appendix 1 Table) rooted to A/Hong Kong/483/1997 was constructed using MEGA version X with 1000 replicates. The scale bar represents the number of substitutions per site. Bootstrap values of $\geq 70\%$ are shown. Human viruses are in bold font. NA subtypes other than N6 and virus strain abbreviations used in this study are indicated in brackets.

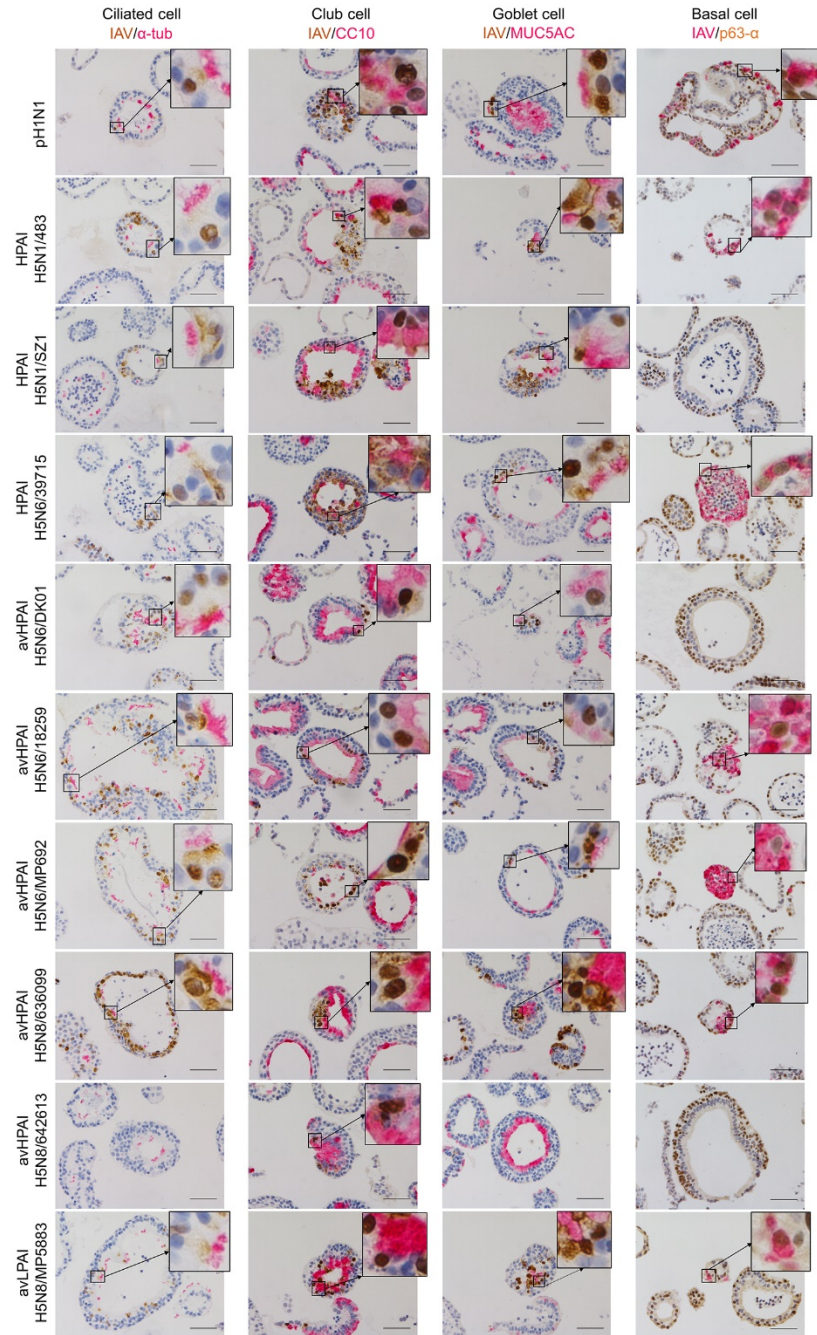
Risk Assessment for Highly Pathogenic Avian Influenza A(H5N6/H5N8) Clade 2.3.4.4 Viruses

Appendix 2

Appendix 2 Table. Molecular features associated with virus receptor-binding preference, mammalian adaptation, pathogenicity, replication efficiency, transmission, and antiviral drug resistance in risk assessment for HPAI influenza A(H5Nx) clade 2.3.4.4 viruses*

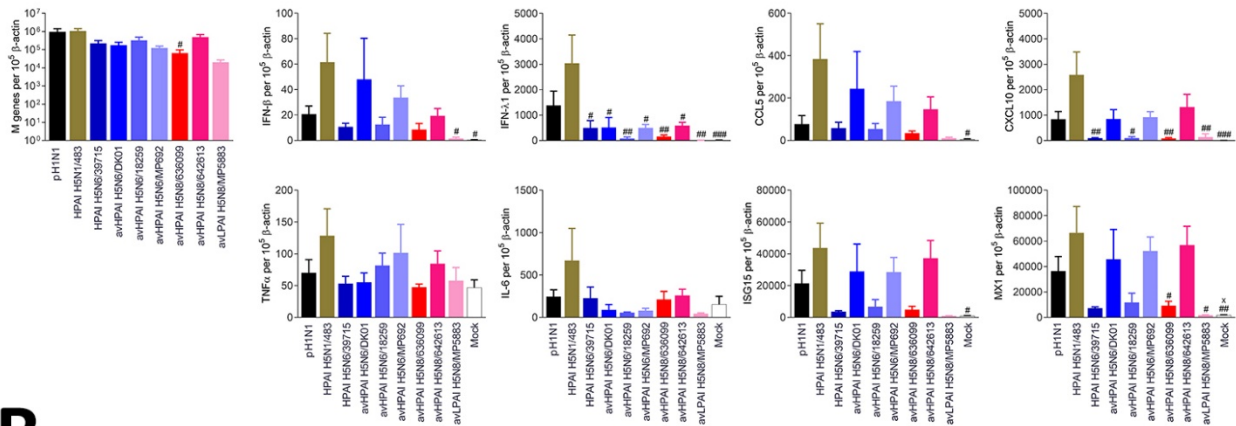
Viruses	Clade	HA†														NA‡	PB2†							PB1†	PB1-F2†	PA†					NP†	M1†			M2†	NS1‡			NS2‡	NA†	M2†
		Cleavage site		Glycosylation site		Receptor binding site							Stalk deletion (aa)	Total aa length	66		7	6	4	6	9	15	16			55	80–84 deletion	92	3	6		18	PDZ ligand domain 227–230	31		56	96	31			
		323–331	154–156	94	12	12	13	14	15	15	18	18																													
HPAI H5N1/483	0	RE RRR K K R ↓G	N S T	N	D	S	S	S	I	K	D	A	T	K	Q	K	S	Yes	90	S	I	M	A	M	N	I	G	F	NO	E	L	I	N	E S E V	I	Y	V	S			
HPAI H5N1/SZ1	2.3.2.1b	I E R R R R K R ↓G	D D A	S	E	L	A	S	L	K	D	A	I	R	K	K	R	Yes	57		V	L	S	L	N	I	E	L	YES	E	F	M	D	E S E V	M	H	V	S			
HPAI H5N6/39715	2.3.4.4	R E R R R K R ↓G	N D T	N	E	L	A	P	T	K	N	A	T	N	K	Q	R	Yes	11		V	L	S	L	N	I	E	L	YES	E	F	M	D	E S E V	M	H	V	S			
avHPAI H5N6/DK01	2.3.4.4h	R E R R R K R ↓G	N D A	N		S	A	A	T	K	S	E	A	D	K	Q	R	Yes	11		V	L	S	L	N	I	E	L	YES	E	F	M	D	E S E V	M	H	V	S			
avHPAI H5N6/18259	2.3.4.4b	R E R R R K R ↓G	N D A	S	E	L	A	P	I	N	N	E	T	N	K	Q	R	No	11		V	L	A	L	N	V	E	L	NO	D	F	M	D		M	H	A	S			
avHPAI H5N6/MP692	2.3.4.4e	R E R R R R K R ↓G	N D A	N	E	L	S	P	T	K	N	A	T	N	K	Q	Q	Yes	90	N	V	L	A	L	N	I	E	L	YES	E	F	M	D	E S E V	M	H	V	N			
avHPAI H5N8/636099	2.3.4.4b	R E K R R R K R ↓G	N D E	S	E	L	A	P	I	K	N	E	T	N	K	Q	R	No	90	S	V	L	A	L	N	V	E	L	NO	D	F	M	D	G S E V	M	H	V	S			
avHPAI H5N8/642613	2.3.4.4b	R E K R R R K R ↓G	N D A	S	E	L	A	P	I	K	N	E	T	N	K	Q	R	No	11		V	L	A	L	K	I	E	L	NO	D	F	M	D	G S E V	M	H	V	S			
avLPAI H5N8/MP5883	NA	R E T R ↓G	N N A	D	D	S	S	S	I	K	D	A	T	K	Q	K	S	No	90	N	V	L	A	L	N	V	E	L	NO	D	F	M	D	E S E V	M	H	V	S			
		Pathogenicity				Receptor binding preference											Virulence, transmission, replication efficiency, and adaptation in mammals														Antiviral drug resistance										

*Boldface indicates previously identified mutations/molecular markers associated with increased viral phenotypic characteristics (bottom row): pathogenicity; binding to α2,6-linked or fucosylated α2,3-linked sialic acid receptors; virulence, transmission, replication efficiency, and adaptation in mammals; and resistance to zanamivir, oseltamivir, amantadine, and rimantadine. ↓ indicates cleavage position. Blank space indicates deleted/missing amino acid(s). Clade classification was done using the Highly Pathogenic H5N1 Clade Classification Tool and according to the phylogenetic relationships of the mature HA1 protein nucleotide sequences (Appendix 1 Figure 2, <https://wwwnc.cdc.gov/EID/article/27/10/21-0297-App1.pdf>). The numbering of the amino acid is relative to A/Vietnam/1203/2004† and A/goose/Guangdong/1/1996‡. Numbering of HA is based on mature sequences without the N-terminal signal peptides. Aa, amino acid; HA, hemagglutinin; HPAI, highly pathogenic avian influenza; LPAI, low pathogenicity avian influenza; NA, not applicable.

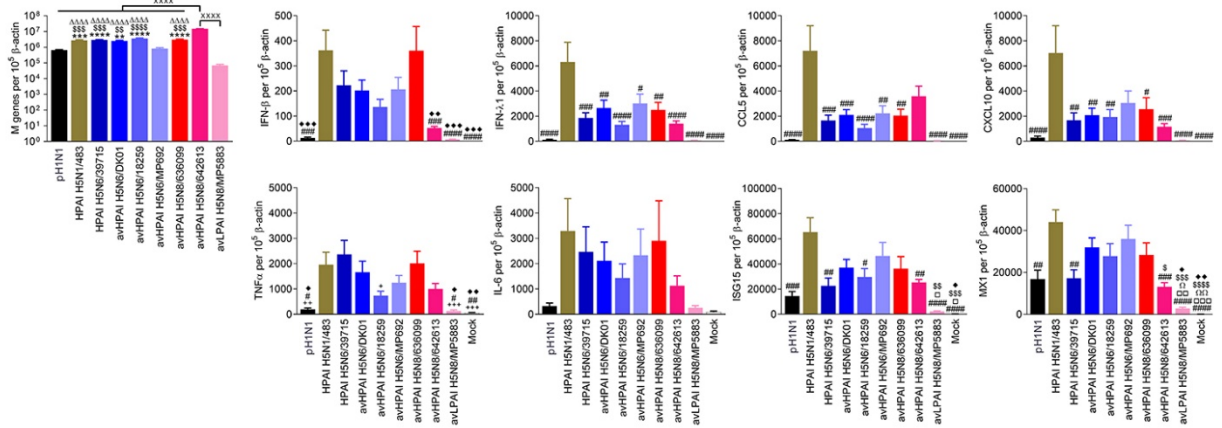


Appendix 2 Figure 1. Representative images of immunohistochemical double staining of influenza A virus (IAV)-infected human airway organoids at 24 or 48 hours post infection (hpi) ($n \geq 3$). Ciliated cells, club cells, and goblet cells were co-stained for IAV nucleoprotein (brown) and acetyl- α -tubulin (α -tub) (pink), SCGB1A1/CC10 (CC10) (pink), and MUC5AC (pink), respectively. Basal cells were co-stained for p63- α (brown) and IAV nucleoprotein (pink). Co-localization regions were enlarged in the upper-right corner. Scale bars = 50 μ m.

A



B



Appendix 2 Figure 2. Cytokine and chemokine mRNA expression in A) human airway organoids infected with 6 log TCID₅₀/ml virus and B) primary human alveolar epithelial cells infected at MOI 2 at 37°C at 24 hours post infection (hpi) (mean±SEM, n ≥ 3). mRNA copy numbers of influenza A virus matrix (M) gene, interferon-β (IFN-β), interferon-λ1 (IFN-λ1), C-C motif chemokine ligand 5 (CCL5), C-X-C motif chemokine 10 (CXCL10), tumor necrosis factor α (TNFα), interleukin 6 (IL-6), interferon-stimulated gene 15 (ISG15), and interferon-induced GTP binding protein Mx1 (MX1) were expressed as per 10⁵ β-actin copies. Statistical significance between mRNA levels was calculated by one-way ANOVA with *Bonferroni* post-tests. **: p ≤ 0.01, ***: p ≤ 0.001, ****: p ≤ 0.0001 (compared to H1N1pdm); #: p ≤ 0.05, ###: p ≤ 0.01, ####: p ≤ 0.001, #####: p ≤ 0.0001 (compared to HPAI H5N1/483); +: p ≤ 0.05, ++: p ≤ 0.01, +++: p ≤ 0.001 (compared to HPAI H5N6/39715); □: p ≤ 0.05, □□: p ≤ 0.01, □□□: p ≤ 0.001 (compared to avHPAI H5N6/DK01); Ω: p ≤ 0.05, ΩΩ: p ≤ 0.01 (compared to avHPAI H5N6/18259); \$: p ≤ 0.05, \$\$: p ≤ 0.01, \$\$\$: p ≤ 0.001, \$\$\$\$: p ≤ 0.0001 (compared to avHPAI H5N6/MP692); *†: p ≤ 0.05, **†: p ≤ 0.01, ***†: p ≤ 0.001 (compared to avHPAI H5N8/636099); x: p ≤ 0.05, xxxx: p ≤ 0.0001 (compared to avHPAI H5N8/642613); ΔΔΔΔ: p ≤ 0.0001 (compared to avLP AI H5N8/MP5883).