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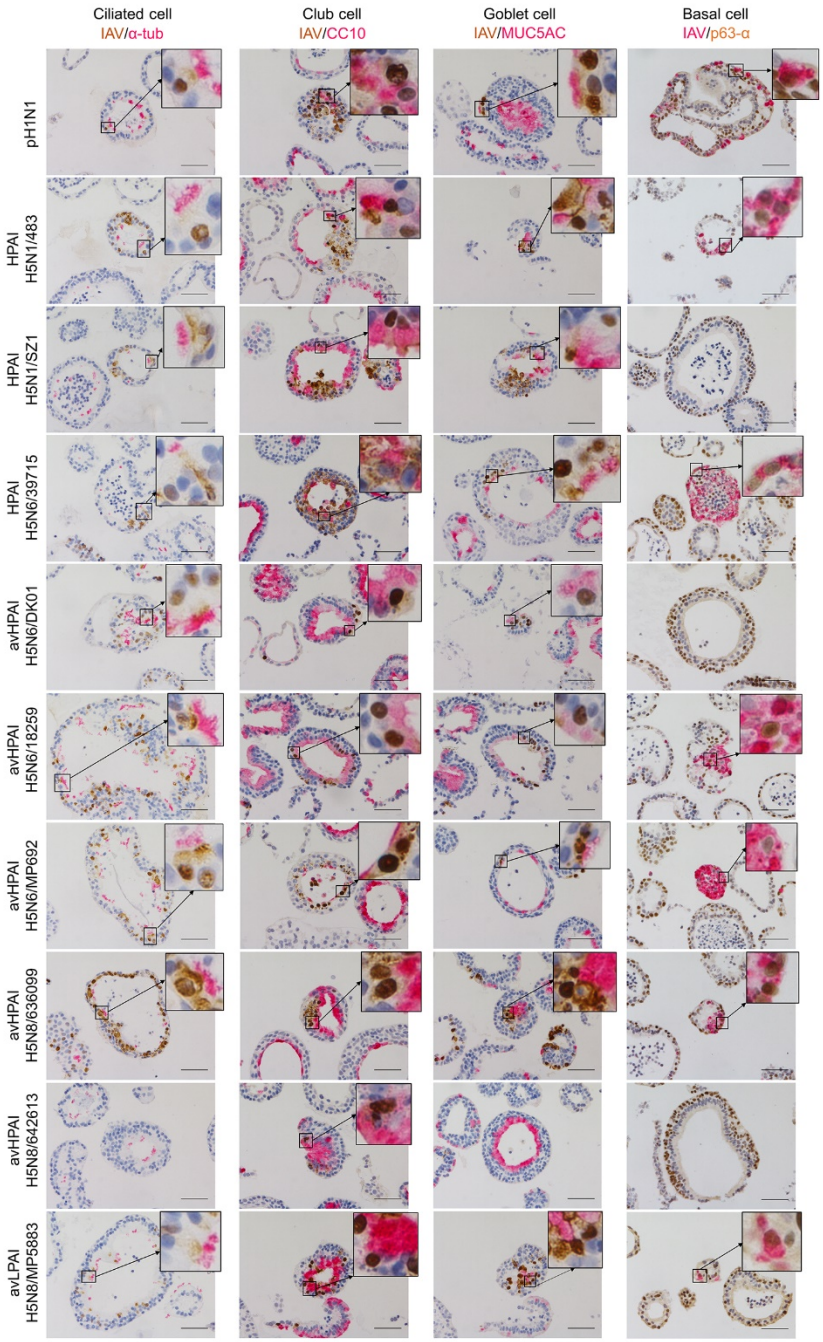
Risk Assessment for Highly Pathogenic Avian Influenza A(H5N6/H5N8) Clade 2.3.4.4 Viruses

Appendix 2

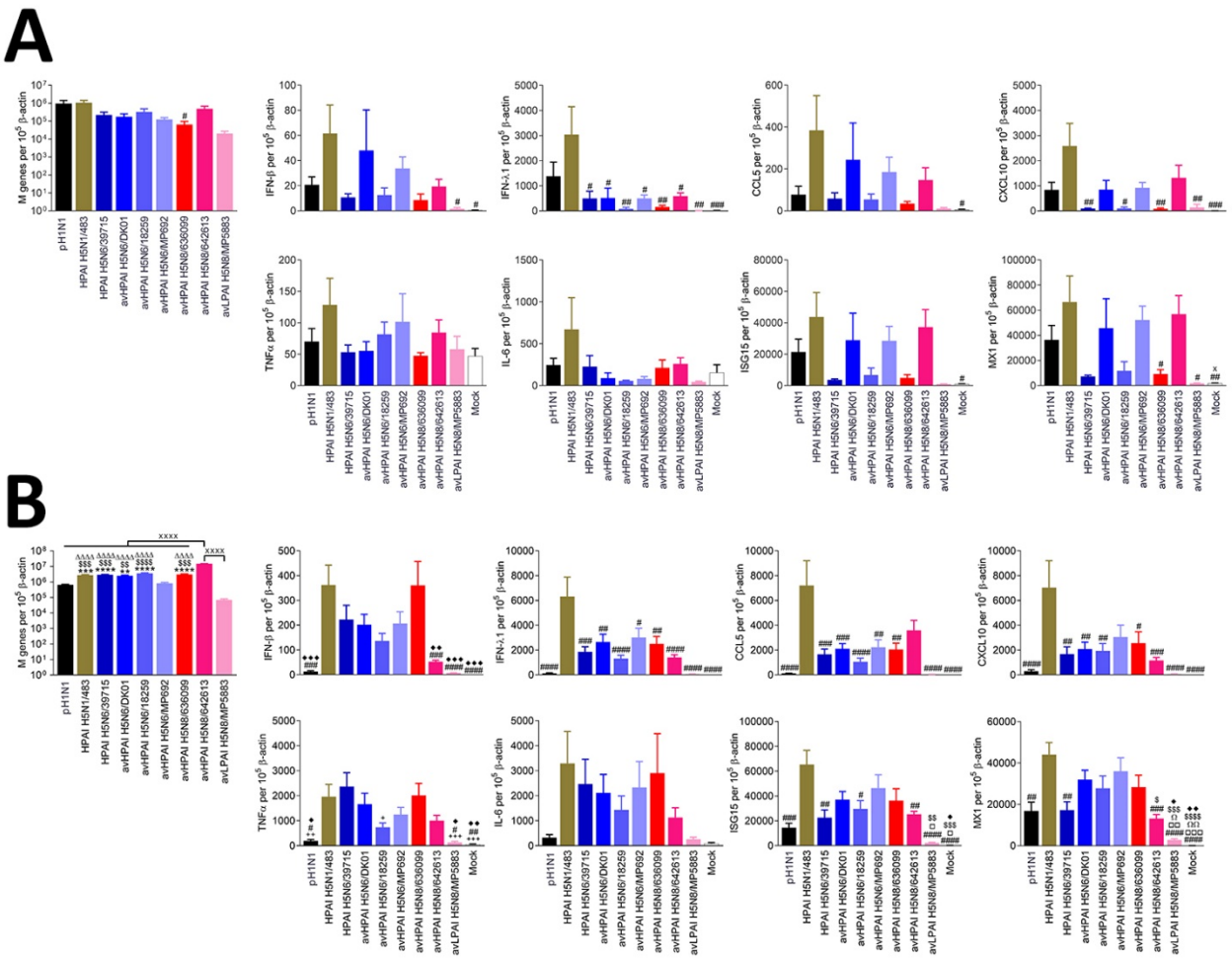
Appendix 2 Table. Molecular features associated with virus receptor-binding preference, mammalian adaptation, pathogenicity, replication efficiency, transmission, and antiviral drug resistance in risk assessment for HPAI influenza A(H5Nx) clade 2.3.4.4 viruses*

		HA†																	NA‡								PB2†									PB1†	PB1-F2†		PA†			NP†		M1†	M2†			NS1‡						NS2‡		NA†	M2†
					Receptor binding site																																																				
		Cleavage site	Glycosylation site		130-loop						190-helix					220-loop			Stalk deletion (aa)																		Total aa length																					PDZ ligand domain 227–230				
Viruses	Clade	323–331	154–156	94	126	129	133	141	151	152	183	185	188	189	192	218	223		64	318	355	627	661	667	701	317		66	76	336	404	436	469	15	16	55	80–84 deletion	92	103	106	189												
HPAI H5N1/483	0	RE**RRRKKR**↓G	NST	**N**	D	S	S	S	I	K	D	A	T	K	Q	K	S	**Yes (50–68)**	M	**K**	**K**	**K**	**T**	**I**	D	**I**	**90**	**S**	**I**	**M**	A	**M**	N	**I**	**G**	**F**	NO	**E**	L	I	**N**	**EPEV**	**I**	**Y**	V	S
HPAI H5N1/SZ1	2.3.2.1b	IE**RRRRKR**↓G	**DDA**	S	E	L	**A**	S	L	K	D	A	**I**	**R**	K	K	**R**	**Yes (49–68)**	**I**	R	R	E	A	V	D	M	57		V	L	**S**	L	N	**I**	E	L	**YES**	**E**	**F**	**M**	D	**ESEV**	M	H	V	S
HPAI H5N6/39715	2.3.4.4	RE**RRRKR**↓G	NDT	**N**	E	L	**A**	P	T	K	N	A	T	N	K	**Q**	**R**	**Yes (58–68)**	**I**	R	**K**	**K**	A	V	D	M	11		V	L	**S**	L	N	**I**	E	L	**YES**	**E**	**F**	**M**	D	**ESEV**	M	H	V	S
avHPAI H5N6/DK01	2.3.4.4h	RE**RRRKR**↓G	ND**A**	**N**		S	**A**	A	T	K	S	E	A	D	K	**Q**	**R**	**Yes (58–68)**	M	R	R	E	S	V	D	M	11		V	L	**S**	L	N	**I**	E	L	**YES**	**E**	**F**	**M**	D	**ESEV**	M	H	V	S
avHPAI H5N6/18259	2.3.4.4b	RE**RRRKR**↓G	ND**A**	S	E	L	**A**	P	I	N	N	E	T	N	K	**Q**	**R**	No	M	R	R	E	A	V	D	M	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	RE**RRRRKR**↓G	ND**A**	**N**	E	L	S	P	T	K	N	A	T	N	K	**Q**	Q	**Yes (58–68)**	M	R	R	E	S	V	D	M	**90**	N	V	L	A	L	N	**I**	E	L	**YES**	**E**	**F**	**M**	D	**ESEV**	M	H	V	**N**
avHPAI H5N8/636099	2.3.4.4b	RE**KRRKR**↓G	NDE	S	E	L	**A**	P	I	K	N	E	T	N	K	**Q**	**R**	No	M	R	R	E	A	V	D	M	**90**	**S**	V	L	A	L	N	V	E	L	NO	D	**F**	**M**	D	**GSEV**	M	H	V	S
avHPAI H5N8/642613	2.3.4.4b	RE**KRRKR**↓G	ND**A**	S	E	L	**A**	P	I	K	N	E	T	N	K	**Q**	**R**	No	M	R	R	E	A	**I**	D	M	11		V	L	A	L	**K**	**I**	E	L	NO	D	**F**	**M**	D	**GSEV**	M	H	V	S
avLPAI H5N8/MP5883	NA	RET**R**↓G	N**NA**	D	D	S	S	S	I	K	D	A	T	K	Q	K	S	No	M	R	R	E	A	V	D	M	**90**	N	V	L	A	L	N	V	E	L	NO	D	**F**	**M**	D	**ESEV**	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 ≥ 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 μm.



Appendix 2 Figure 2. Cytokine and chemokine mRNA expression in A) human airway organoids infected with 6 log $TCID_{50}$/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 10^5 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 avLPAI H5N8/MP5883).