Tropism and innate host responses of influenza A/H5N6 virus: an analysis of *ex vivo* and *in vitro* cultures of the human respiratory tract

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Well adapted to infect the human respiratory tract, influenza A/H5N6 viruses pose a significant public health threat http://ow.ly/c77e308z6Ju

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ABSTRACT Since their first isolation in 2013, influenza A/H5N6 viruses have spread amongst poultry across multiple provinces in China and to Laos, Vietnam and Myanmar. So far, there have been 14 human H5N6 infections with 10 fatalities.

We investigated the tropism, replication competence and cytokine induction of one human and two avian H5N6 isolates in *ex vivo* and *in vitro* cultures derived from the human respiratory tract. Virus tropism and replication were studied in *ex vivo* cultures of human nasopharynx, bronchus and lung. Induction of cytokines and chemokines was measured *in vitro* in virus-infected primary human alveolar epithelial cells.

Human H5N6 virus replicated more efficiently than highly pathogenic avian influenza (HPAI) H5N1 virus and as efficiently as H1N1pdm in *ex vivo* human bronchus and lung and was also able to replicate in *ex vivo* cultures of human nasopharynx. Avian H5N6 viruses replicated less efficiently than H1N1pdm in human bronchial tissues and to similar titres as HPAI H5N1 in the lung. While the human H5N6 virus had affinity for avian-like receptors, the two avian isolates had binding affinity for both avian- and human-like receptors. All three H5N6 viruses were less potent inducers of pro-inflammatory cytokines compared with H5N1 virus.

Human H5N6 virus appears better adapted to infect the human airways than H5N1 virus and may pose a significant public health threat.

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Introduction

Highly pathogenic avian influenza (HPAI) virus H5N1 was first isolated in geese in 1996 in Guangdong Province, China [1]. Since then, this virus lineage has spread to affect poultry in multiple countries across Asia and Africa and, as of July 2016, there have been 854 human cases with 450 deaths [2]. These Asian HPAI H5 subtype viruses continue to evolve and reassort to generate multiple virus clades and gene constellations. Recently, genetic reassortment within viruses in clade 2.3.4.4 resulted in the emergence of multiple novel H5 virus subtypes including H5N2, H5N5, H5N6 and H5N8. Some of these viruses have spread to cause infections in poultry or wild birds in Asia, Europe and North America [3, 4]. H5N6 virus was first detected in poultry in China in 2013 and has now spread to over 15 provinces in China, and to Vietnam, Laos and Myanmar [5]. The first human H5N6 infection was reported in April 2014 in Sichuan, China and had a fatal outcome [6, 7]. So far, 14 human infections with H5N6 have been reported and only four patients survived [8, 9]. The source of infection was mainly exposure to infected poultry and there is no evidence so far of human-to-human transmission. Patients infected with H5N6 presented with high fever, chills, cough and breathing difficulties. They progressed to develop severe pneumonia, multiple organ failure and acute respiratory distress syndrome (ARDS) [9, 10]. In the one autopsy report available, the most prominent histopathological feature was diffuse alveolar damage with pulmonary vasculitis; however, virus antigen was also detected in the intestinal tract, kidney, spleen and brain [9]. As the respiratory tract is the initial site of virus infection and the main site of pathology, virus tropism in the human respiratory tract is of relevance in assessing influenza H5N6 viruses for zoonotic and pandemic risk.

In this study, we compared the virus tropism and replication efficiency of human A/Guangzhou/39715/2014 (H5N6) virus and two avian H5N6 viruses with those of HPAI H5N1 virus and 2009 pandemic H1N1 virus in *ex vivo* cultures of human nasopharynx, bronchus and lung. The induction of pro-inflammatory cytokines and chemokines induced by H5N6 virus infection of human alveolar epithelial cells was investigated and compared with HPAI H5N1.

Materials and methods

Viruses

A/Guangzhou/39715/2014 (H5N6) virus, abbreviated as H5N6/39715 (GenBank accession number: KP765785 to KP765792) [8], was isolated from the throat swab of a 59-year-old male patient on day eight of illness. This patient survived infection and was discharged on day 58 [9]. Phylogenetic analysis had previously revealed that the haemagglutinin (HA) gene of H5N6/39715 belonged to the H5 clade 2.3.4.4 H5, the neuraminidase (NA) gene originated from influenza A H6N6 viruses circulating among domestic ducks in China, and the internal genes were closely related to H5N6 viruses found in poultry and chickens in China and Laos [11]. The cleavage site of the HA gene possessed multiple basic amino acids compatible with HPAI [12] but mutations known to be associated with enhanced binding to human-like receptors were not found [13]. The deletion of 11 amino acid residues at positions 59–69 in the NA gene was noted, an alteration associated with the adaptation of influenza viruses from aquatic birds for replication in terrestrial poultry. Furthermore, the polymerase basic 2 protein (PB2) gene of this human virus had acquired the mammalian adaptation marker E627K, known to increase virulence and pathogenicity in mice [11].

The two other H5N6 influenza viruses studied were isolated from wild bird carcasses in Hong Kong. These were 1) A/Oriental Magpie Robin/HK/6154/2015 (avH5N6/6154) and 2) A/Peregrine Falcon/HK/4955/2015 (avH5N6/4955) (GenBank accession number: KX638404–KX638420). In addition, an HPAI H5N1 virus isolated from a fatal human case in Hong Kong (A/Hong Kong/483/1997 (H5N1/483); GenBank accession number: GU052096–GU052104), a more recent human HPAI H5N1 isolate (A/Shenzhen/1/2011 (H5N1/SZ1)), and a pandemic H1N1 virus (A/Hong Kong/415742/2009 (H1N1pdm); GISAID accession number: EPI307225–EPI307233) were used for comparison. The three H5N6 viruses were first isolated by inoculation into special pathogen-free embryonated chicken eggs while the other viruses were isolated in Madin–Darby canine kidney (MDCK) cells. Virus stocks used were prepared in MDCK cells and viral titres were determined as previously described [14]. All experiments were performed in a biosafety level-3 facility.

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Conflict of interest: None declared.

Ex vivo *cultures*

Fresh bronchus, lung and nasopharynx were obtained from tissue removed as part of the routine clinical care of patients in Hong Kong undergoing surgical resection. After diagnostic requirements were met, any residual excess tissue was retrieved and used in this study. Informed consent was obtained from all subjects 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 14-119). Methods of culture, infection and analysis were performed as previously described [15].

In vitro *cultures*

Primary human alveolar epithelial cells (AECs) were isolated and used for infection as previously described [16]. Briefly, AECs were infected with influenza A viruses for 1 h at 37 °C, either at a multiplicity of infection (MOI) of 0.01 (to allow multiple cycles of infection and thus the investigation of viral replication kinetics) or a MOI of 2.0 (to ensure the synchronous infection of most cells in the culture and allow the analysis of cytokine and chemokine expression). Mock-infected cells served as negative controls. The virus inoculum was removed and the cells were washed with warm PBS and replenished with small airway growth medium containing supplements (SABM™ medium; Lonza, USA) together with penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$). The viral titre of the culture supernatant was determined by 50% tissue culture infectious dose ($TCID_{50}$) assay as previously described [16]. Cell lysates were collected at 24 h post-infection for measurement of mRNA expression of cytokines while cells were collected at 24 h post-infection and fixed in 4% paraformaldehyde for immunofluorescence staining of viral protein.

Thermal inactivation assay

Virus inoculum (1 mL) was added to 24-well tissue culture plates with starting concentrations of 10^4, 10^3 and 10^2 $TCID_{50} \cdot mL^{-1}$. Supernatant was collected at 1, 24, 48 and 72 h post-infection for the calculation of viral titres by $TCID_{50}$ assay to assess the loss of virus viability in the absence of virus replication.

Real-time PCR assay

The RNA of infected cells was extracted using a MiniBEST universal RNA extraction kit (Takara Biotechnology, Dalian, China). cDNA was generated from mRNA using a PrimeScript™ real-time PCR kit with $oligo(dT)_{12–18}$ (Takara Biotechnology) according to the manufacturer's protocol. mRNA expression of target genes was carried out using an ABI ViiA™ 7 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The gene expression profiles of cytokines and chemokines were quantified and normalised with β-actin at the respective times. The primers used in these assays have been published previously [16–18].

Bead-based immunoassay

The concentration of cytokines and chemokines in the culture supernatants were determined using a multiplexed bead-based immunoassay (BD™ Cytometric Bead Array (CBA); BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol (see supplementary material). The samples were analysed using a BD LSRFortessa™ Analyser (BD Biosciences). Standard curves for the cytokines and chemokines were derived and the concentrations with respect to fluorescence intensity were calculated using FlowJo® version 7.6.1 (FlowJo, Ashland, OR, USA).

Desialylation–haemagglutination assay

The effect of desialylation of TRBCs on virus haemagglutination was investigated. TRBCs treated with sialidase DAS181 (NexBio, San Diego, CA, USA), which removed both α-2,3-linked and α-2,6-linked sialic acids (SAs), or Glyko® Sialidase S™ (Prozyme, Hayward, CA, USA), which removed only α-2,3-linked SAs, were compared with untreated TRBCs as controls (see supplementary material).

Saturation transfer difference nuclear magnetic resonance spectrometry

Saturation transfer difference NMR spectrometry (STD-NMR) was performed using paraformaldehyde-fixed viruses and SA receptor analogs (3′-sialyl-*N*-acetyllactosamine (3′SLN) and 6′-sialyl-*N*-acetyllactosamine (6′SLN)) with analysis on a 750 MHz spectrometer (Varian, Palo Alto, CA, USA) (see supplementary material).

Immunohistochemical staining

The human tissues were embedded in paraffin and stained for influenza viral protein (see supplementary material). 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 NP-specific mouse monoclonal antibody HB65 (EVL, Woerden, The Netherlands) and a biotinylated secondary antibody. The bound antibodies were visualised with a Strep-ABC complex and an AEC substrate kit.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA). Viral replication kinetics between experimental groups was compared using one-way analysis of variance (one-way ANOVA), while that of mRNA and also protein expression of cytokines and chemokines was compared using Student's t-test.

Results

Mammalian adaptation markers in the viruses investigated

While the two avian H5N6 viruses had PB2 genes with 627E residues, the human H5N6/39715 virus and H5N1/483 virus had the amino acid substitution PB2 E627K associated with mammalian adaptation (table 1). No other compensatory mammalian adaptation markers, such as PB2 D701N, were found. In comparison with the clade 0 H5N1 viruses used as references, the HA gene of the H5N6 viruses exhibited differences in the amino acid residues in the receptor-binding site and in the 158–160 glycosylation motif. The two avian H5N6 viruses showed the loss of a putative N-linked glycosylation site at HA residues 158–160 while the human H5N6/39715 and H5N1/483 viruses retained it. All three H5N6 viruses had a deletion in the stalk of the NA gene as did the H5N1/483 virus (table 1). The NA H275Y mutation, which confers oseltamivir resistance, was not observed in either human or avian H5N6 viruses. However, the M2 S31N mutation, which confers amantadine resistance, was observed in avH5N6/4955.

Comparative virus replication competence and tropism of H5N6, H5N1 and pandemic H1N1 viruses

The human H5N6/39715 virus replicated efficiently in *ex vivo* cultures of human bronchus and lung to similar levels as the H1N1pdm virus at all time points (figures 1a–1f). It had a significantly higher replication competence than the human HPAI H5N1 viruses (H5N1/483 and H5N1/SZ1) in human bronchus cultures (figures 1a, 1c and 1e). In the lung, human H5N6/39715 virus replicated to significantly higher titre than the HPAI H5N1 viruses at 24 h post infection (figures 1b, 1d and 1f).

The two avian H5N6 viruses were able to infect *ex vivo* human lung and bronchus cultures but there was a trend towards lower replication than the human H5N6/39715 virus. Furthermore, both avian viruses exhibited a trend towards lower replication capacity than the HPAI H5N1/483 virus in bronchus tissue (figures 1a and 1c), similar replication competence as the H5N1/483 virus in lung tissue (figures 1d and 1f), and lower replication competence than the H1N1pdm virus in both human bronchus and lung at 24 h post-infection (figures 1e and 1f).

Immunohistochemical staining of human bronchial epithelium showed the most extensive evidence for virus antigen positive cells with the H1N1pdm virus, moderate levels of infection with the three H5N6 viruses and least evidence of infection with either H5N1 virus (figure 2a). In the lung, human H5N6/39715 virus and the two H5N1 viruses (483 and SZ1) had a similar extent of infection, which was higher than that of the two avian H5N6 viruses and the H1N1pdm virus (figure 2b).

The three H5N6 viruses infected both ciliated and non-ciliated cells in *ex vivo* bronchial tissue and predominantly type-II AECs in *ex vivo* lung tissue (both H5N1 viruses predominantly infected type-II AECs in lung tissue). Viral protein staining was more intense in H5N6/39715 infected lung tissue at 48 h and 72 h post-infection when compared to H5N1/483 infected lung tissue. Furthermore, H5N1/SZ1 infected tissue had only sparse positive cells at 48 h and 72 h post-infection (see supplementary figure S1). These observations were consistent with replication kinetics showing that the H5N1/483 and H5N6/39715 viruses replicated more efficiently than the avian H5N6 viruses and the H5N1/SZ1 virus in *ex vivo* human lung

TABLE 1 Molecular features associated with viral pathogenicity, transmissibility and antiviral resistance

Virus	Clade	HA							NA		PB2		M2
		Cleavage site	Glycosylation motif	Receptor-binding site									
			158–160[#]	187	193	196	133–137	225–228	59–69[¶]	275[+]	627	701	31[§]
H5N1/483	0	RERRRKKRG	NST	A	K	Q	SGVSS	GQSG	Yes	H	K	D	S
avH5N6/6154	2.3.4.4	RERRRKR↓G	NDA	N	N	K	LGVSA	GQRG	Yes	H	E	D	S
avH5N6/4955	2.3.4.4	RERRRKR↓G	NDA	N	N	K	SGVSA	GQSG	Yes	H	E	D	N
H5N6/39715	2.3.4.4	RERRRKR↓G	NDT	N	N	K	LGVSA	GQRG	Yes	H	K	D	S
H1N1pdm	N/A	IQSR↓G	ENS	T	S	Q	KGVTA	DQEG	No	H	E	D	S

HA: haemagglutinin; NA: neuraminidase; PB2: polymerase basic 2; M2: matrix-2; N/A: not applicable. [#]: H3 numbering; [¶]: deletion in the stalk of NA at this position; [+]: molecular markers of oseltamivir-resistance. [§]: molecular markers of amantadine-resistance.

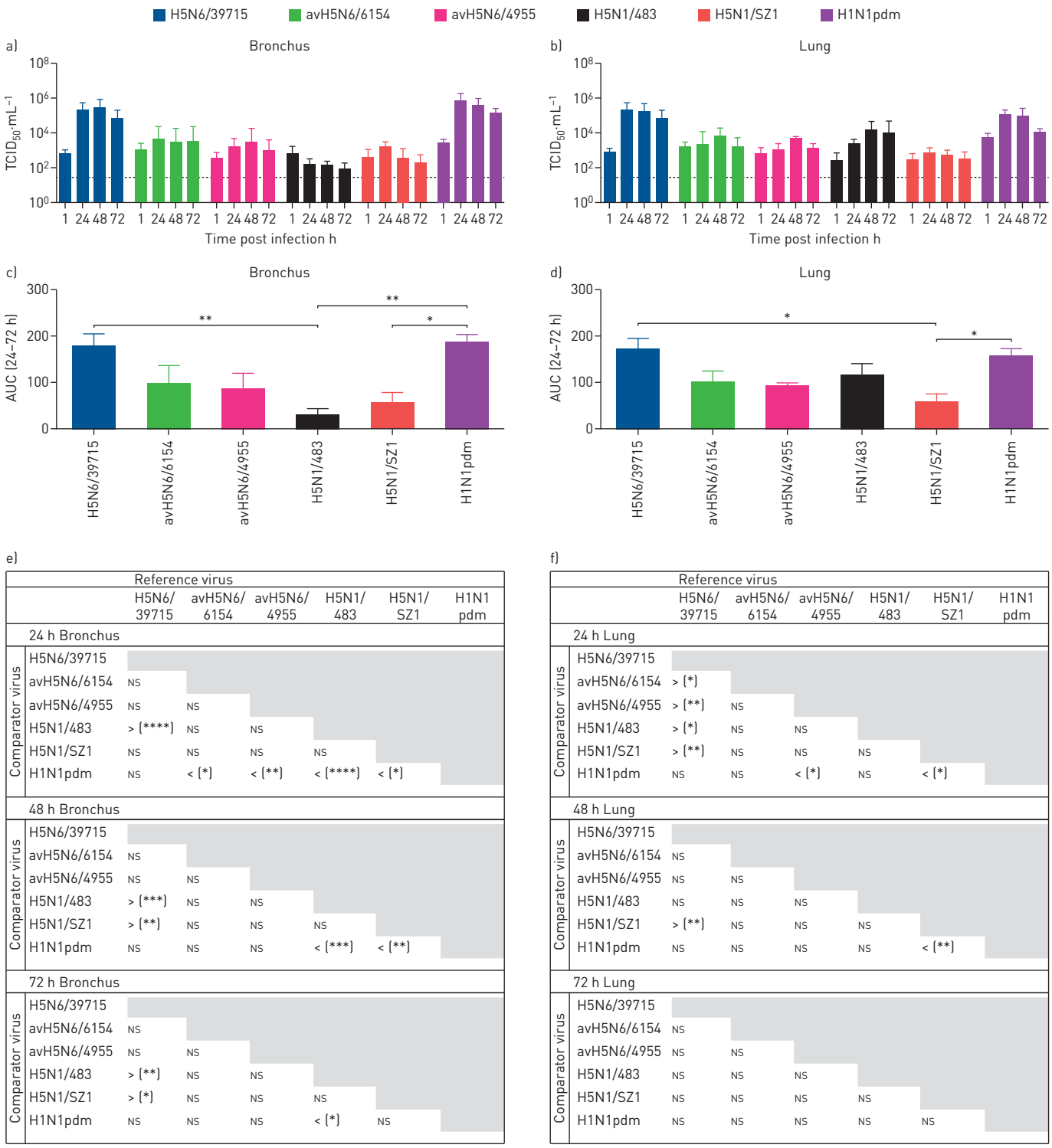
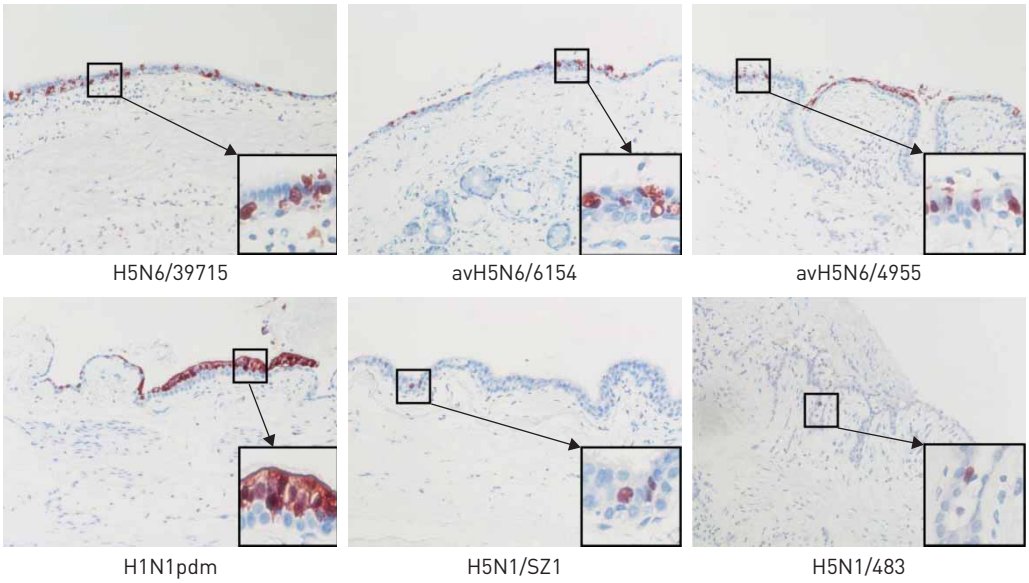
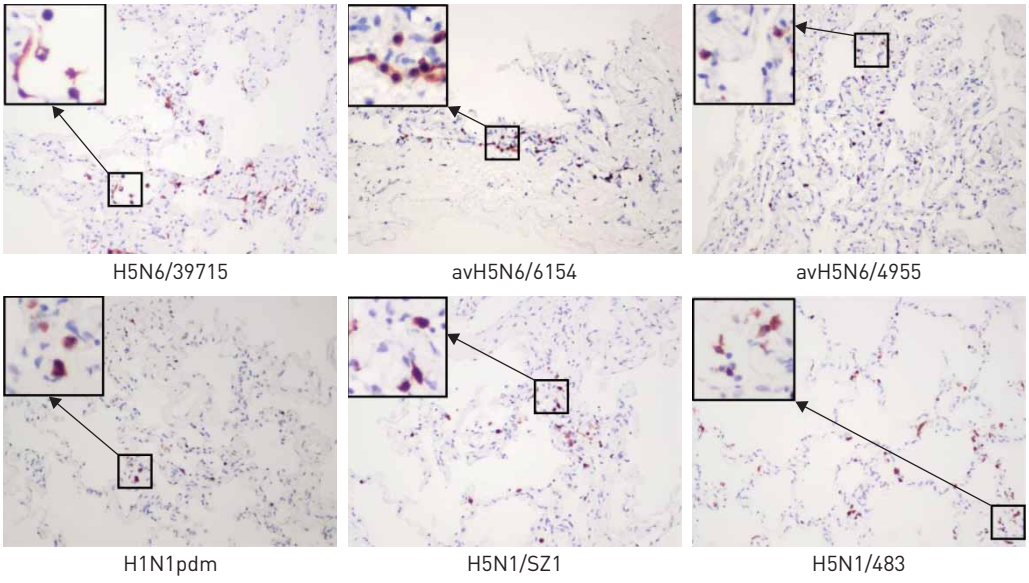


FIGURE 1 Illustration of viral replication kinetics for influenza A viruses in *ex vivo* cultures of human bronchus (a) and lung (b) infected at 37 °C (10^6 $TCID_{50}$·mL^{-1}). The viral titre in the supernatant was determined by $TCID_{50}$ assay and data were pooled from at least three independent experiments and shown as the mean±SEM. The horizontal dotted lines denote the limit of detection in the $TCID_{50}$ assay. The AUC over the period 24 h–72 h post-infection is shown for both bronchus (c) and lung (d). Data were pooled from at least three independent experiments and shown as the mean±SEM calculated by one-way ANOVA. The statistical significance between virus titres at each time point post-infection is shown for both bronchus (e) and lung (f) as calculated by two-way ANOVA. AUC: area under curve; $TCID_{50}$: 50% tissue culture infectious dose; ANOVA: analysis of variance. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; >: reference virus value is significantly higher than the comparator virus; <: reference virus value is significantly lower than the comparator virus; NS: nonsignificant.

a) Bronchus



b) Lung



c)

Virus	Bronchus	Lung
H5N6/39715	++	++
avH5N6/6154	++	+
avH5N6/4955	++	+
H5N1/483	sparse	++
H5N1/SZ1	sparse	++
H1N1pdm	+++	+

FIGURE 2 Tissue tropism of influenza A viruses in *ex vivo* cultures of human bronchus (a) and lung (b). Formalin-fixed, paraffin-embedded sections of human bronchus and lung were subjected to immunohistochemical staining at 24 h post-infection with H5N6/39715, avH5N6/6154, avH5N6/4955, H5N1/483, H5N1/SZ1 and H1N1pdm viruses. Sections were stained with a monoclonal antibody against the influenza nucleoprotein with positive cells identified by a red-brown colour. Tissue tropism of influenza virus infection in *ex vivo* cultures of human bronchus and lung was also assessed by immunohistology (c). Magnification: ×200.

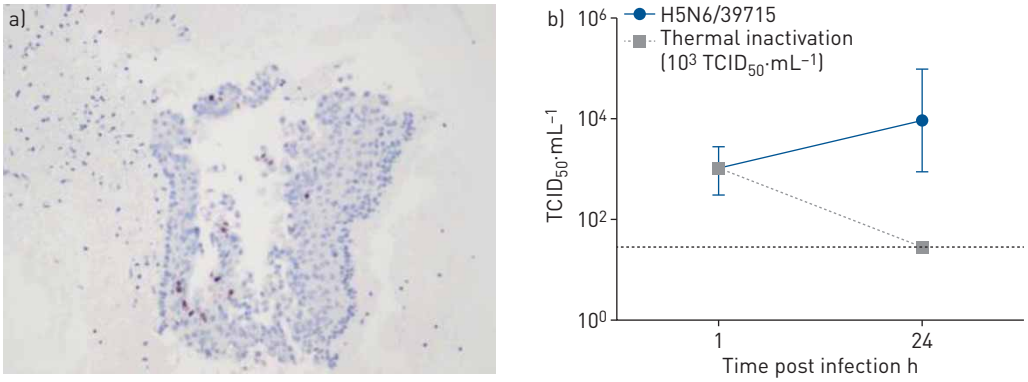


FIGURE 3 Tissue tropism and viral replication kinetics of influenza A H5N6 virus in *ex vivo* cultures of nasopharyngeal tissue. The *ex vivo* cultures of human nasopharyngeal tissue were infected with H5N6/39715 virus (10^6 $TCID_{50}$·mL^{-1}) at 33 °C. Formalin-fixed, paraffin-embedded sections of this tissue were subjected to immunohistochemical staining with a monoclonal antibody against the influenza nucleoprotein at 24 h post-infection. Positive cells were identified as a red-brown colour (a), magnification: ×200. The viral titres of the supernatants from infected cultures and wells without tissue (thermal inactivation) were determined by $TCID_{50}$ assay. Data were pooled from at least three independent experiments and shown as mean±SEM. The horizontal dotted line denotes the limit of detection in the $TCID_{50}$ assay (b).

cultures. The viral titres in the supernatant were confirmed by thermal inactivation assay using tissue-free virus inoculum (supplementary figure S2). Human H5N6/39715 virus also had the ability to infect and productively replicate in *ex vivo* human nasopharyngeal tissue cultures at 33 °C (figures 3a and 3b).

Receptor binding profiles of H5N6 viruses

The treatment of TRBCs with Glyko® Sialidase S™ (Prozyme) led to the complete removal of the α-2,3-link of SA with galactose and thus prevented the haemagglutination of H5N6/39715, H5N1/483 and H5N1/SZ1; however, the haemagglutination of the two avH5N6 isolates and H1N1pdm was not affected (table 2). Treatment of TRBCs with sialidase DAS181 (NexBio) cleaves both the α-2,3- and α-2,6-links of SA with galactose and thus prevents the haemagglutination of all influenza viruses tested. These data suggest that the H5N6/39715 and H5N1 viruses predominantly bind α-2,3-glycans while the avH5N6 isolates and H1N1pdm can bind to glycans other than those with α-2,3-links to SA. However, comparison of haemagglutination using TRBCs and Horse red blood cells (HRBCs), which consist mainly of α-2,3-linked SA [19], indicated a reduction in haemagglutination for H5N6/39715 and H5N1/483 suggesting that these two viruses have higher affinity for the α-2,3-linked SA in TRBCs than that in HRBCs (table 2). Similar levels of haemagglutination were observed for the two avH5N6 isolates in both TRBCs and HRBCs. These results imply that while the two avH5N6 viruses can bind to α-2,6-linked SA in sialidase S™ treated TRBCs, they can also bind to the α-2,3-linked SA in HRBCs and thus may have dual-affinity for both types of linkage.

STD-NMR was performed to validate the receptor binding profile of the terminated H5N6 viruses. Significant binding signals of HA against the 3′SLN and 6′SLN mixture was shown by the STD spectrum of H5N6/39715 (figure 4a). The STD signals for 3′SLN (NeuH3ax and NeuH3eq at 1.47 ppm and

TABLE 2 Effect of desialylation on virus haemagglutination of Turkey red blood cells (TRBCs) and Horse red blood cells (HRBCs).

Virus	0.5% TRBCs			1% HRBCs
	Untreated	Sialidase S™	DAS181	Untreated
H5N6/39715	256	0	0	128
avH5N6/6154	128	128	0	128
avH5N6/4955	128	128	0	128
H5N1/483	128	0	0	32
H5N1/SZ1	128	0	0	NA
H1N1pdm	128	128	0	0

The reciprocal of the haemagglutination titre is denoted. Three independent experiments were performed with identical results. NA: result not available.

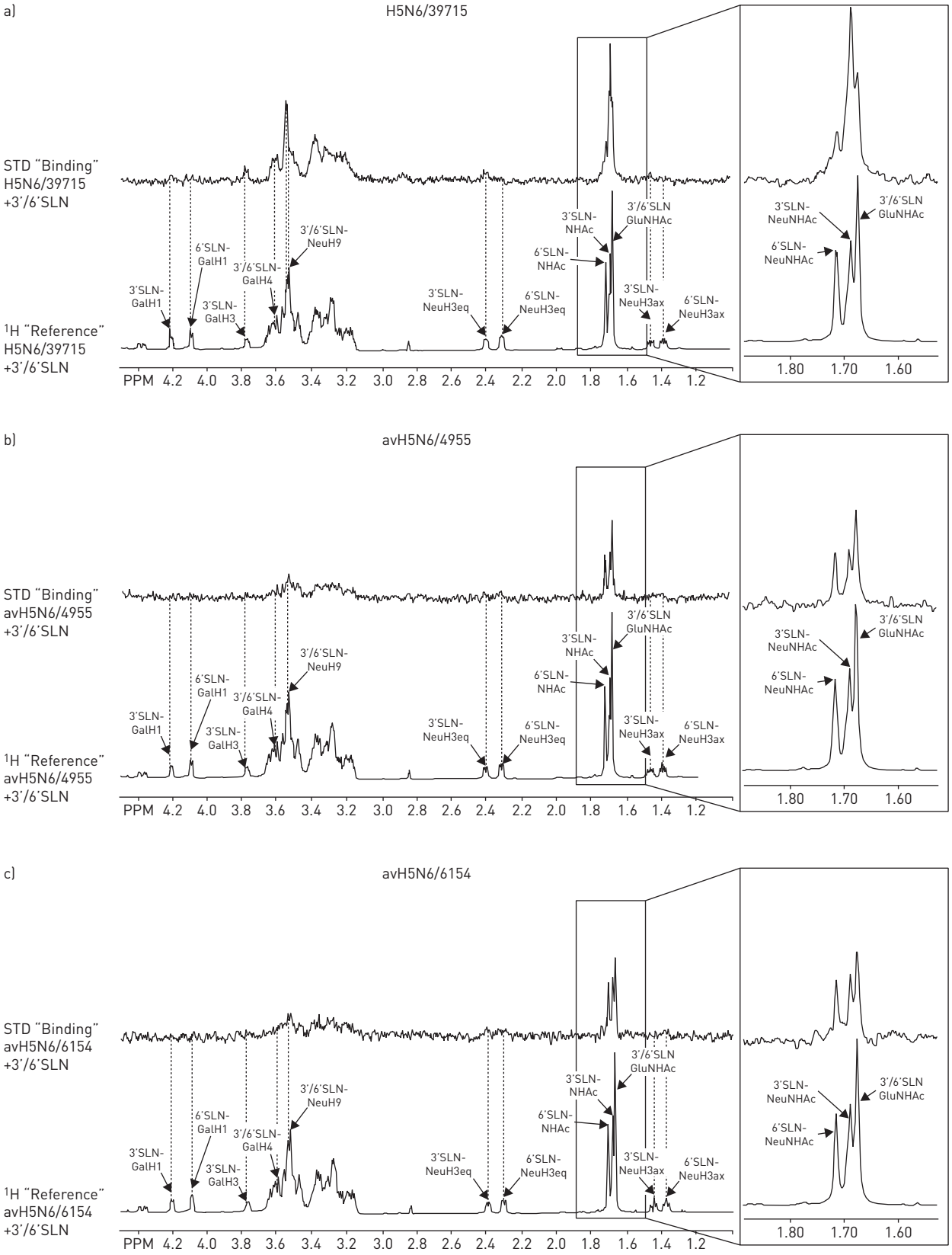


FIGURE 4 Differential receptor binding affinity of influenza H5N6 viruses by analysis using saturation transfer difference NMR spectrometry (STD-NMR). STD-NMR and ^{1}H NMR spectra are shown in the upper and lower panels of each example, respectively. The binding activity of haemagglutinin (HA) against 3′-sialyl-*N*-acetyllactosamine (3′SLN) and 6′-sialyl-*N*-acetyllactosamine (6′SLN) is shown for H5N6/39715 (a), avH5N6/4955 (b) and avH5N6/6154 (c). Signals of some representative hydrogen atoms are indicated in the spectra and the spectral region from 1.5–1.9 ppm is expanded to display the NHAc signals of different moieties in the right panel of each figure. NHAc: acetylamino; Neu: neuraminic acid; Gal: Galactose; GluNHAc: *N*-acetylglucosamine; eq: equatorial; ax: axial.

2.40 ppm) were stronger than those of 6′SLN (NeuH3ax and NeuH3eq at 1.39 ppm and 2.31 ppm). The STD signal of NeuNHAc from 6′SLN binding at 1.72 ppm was calculated to be 33% of the NeuNHAc signal from 3′SLN binding at 1.69 ppm, indicating that the HA from H5N6/39715 has a preference for binding 2,3-sialyl glycans as compared with the 2,6-counterparts.

When compared to H5N6/39715, the STD spectra of both avH5N6 viruses displayed weaker HA binding to the SLN mixture (figures 4b and 4c). Similar signals were observed for NeuH3eq (2.40 ppm and 2.31 ppm) and NeuNHAc (1.69 ppm and 1.72 ppm) of 3′SLN and 6′SLN. Therefore, the STD-NMR results confirmed that the two avH5N6 viruses (avH5N6/4955 and avH5N6/6154) displayed dual binding affinity to both 3′SLN and 6′SLN (*i.e.* the α-2,3- and α-2,6-linked SAs), while the human H5N6/39715 virus showed a clear preference for 3′SLN (the α-2,3-linked SA). Furthermore, the overall binding avidity of the avian H5N6 viruses was lower than that of the human H5N6 virus.

Pro-inflammatory cytokine induction profiles of H5N6 viruses

All the influenza viruses tested infected >80% of AECs (at MOI 2) by 24 h post-infection, as determined by immunofluorescence staining. The human H5N6 virus (H5N6/39715) appeared to replicate at a faster rate than the other viruses, followed by HPAI H5N1/483, as reflected in immunofluorescence micrographs showing that the nucleoprotein (NP) and matrix (M) proteins spread out from nucleus to the cytoplasm at 24 h post-infection in AECs (supplementary figure S3). In infected AECs (MOI 0.01), human H5N6/39715 virus replicates to significantly higher titres when compared to all other viruses tested, including HPAI H5N1/483 and H1N1pdm (figure 5). The avH5N6/4955 and H1N1pdm viruses replicated to significantly lower titres than H5N1/483, while avH5N6/6154 replicated to similar titres as H5N1/483 and higher titres than H1N1pdm.

The mRNA levels of influenza matrix (M) gene expression were similar among all the viruses tested at 24 h post-infection (figure 6a). H5N1/483 and H1N1pdm served as controls of high and low pro-inflammatory cytokine and chemokine inducing viruses, respectively. The human H5N6 virus induced significantly higher levels of interferon-β (IFN-β) and CC chemokine ligand 2 (CCL2) than H1N1pdm (figures 6b and 6e). Similarly, avH5N6/6154 induced significantly higher levels of IFN-β, CC chemokine ligand 5 (CCL5), and CCL2 than H1N1pdm (figures 6b, 6d and 6e), similar levels of IFN-β, CCL5 and CXC chemokine 10 (CXCL10) as H5N1/483 (figures 6b, 6d and 6f), and higher levels of CCL2 than H5N1/483 (figure 6e). In addition, avH5N6/4955 infection led to higher induction of IFN-β, interferon-λ1 (IFN-λ1), CCL5 and CCL2 than H1N1pdm (figures 6b–6e). H5N1/483 induced significantly more CCL5 protein in culture supernatants compared to the other viruses tested (figure 6h). Furthermore, H5N1/483 induced significantly more CCL2 and CXCL10 than the human H5N6/39715 and H1N1pdm viruses and the avian avH5N6/4955 virus (figures 6i and 6j). Finally, avH5N6/6154 induced similar levels of CCL2 and CXCL10 protein as H5N1/483 virus and significantly more CCL2 and CXCL10 protein secretion than human H5N6/39715 and H1N1pdm viruses and avian avH5N6/4955 virus (figures 6i and 6j).

Discussion

The human H5N6/39715 isolate and H1N1pdm had similar replication competences and replicated to higher titres than the HPAI H5N1 isolate in *ex vivo* human bronchus and lung tissues. Unlike the human

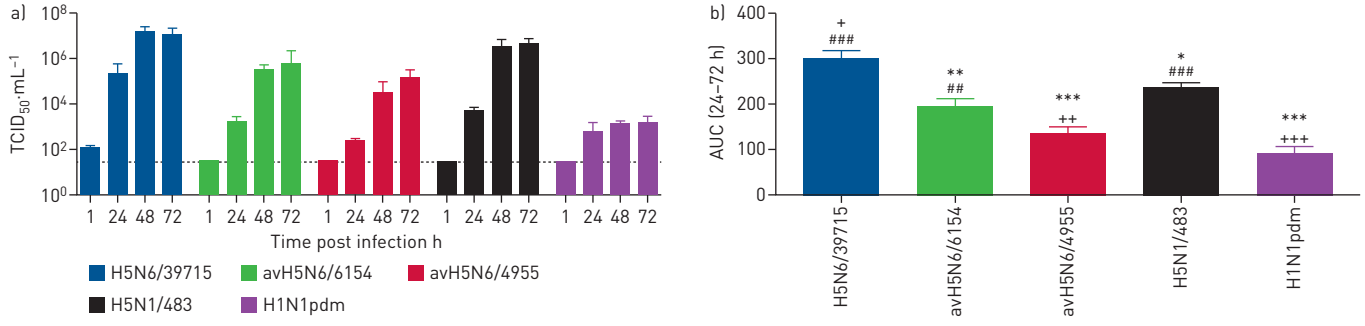


FIGURE 5 Viral replication kinetics of influenza A viruses in *in vitro* cultures of human alveolar epithelial cells (AECs). AECs were infected with the indicated viruses at a multiplicity of infection (MOI) of 0.01 and cultured at 37 °C for 72 h. Culture supernatants were harvested at the indicated times and virus titres were measured by 50% tissue culture infectious dose ($TCID_{50}$) assay. Results (a) are pooled from three independent experiments and shown as mean±SEM. The horizontal dotted line denotes the limit of detection in the $TCID_{50}$ assay. The area under curve (AUC) over a 24–72 h period is pooled from three independent experiments and shown as mean±SEM (b). *: p<0.05; **: p<0.01; ***: p<0.001 (compared to H5N6/39715); $^{\#\#}$: p<0.01; $^{\#\#\#}$: p<0.001 (compared to H1N1pdm); $^{+}$: p<0.05; $^{++}$: p<0.01; $^{+++}$: p<0.001 (compared to H5N1/483). Statistical significances were calculated by one-way analysis of variance (one-way ANOVA).

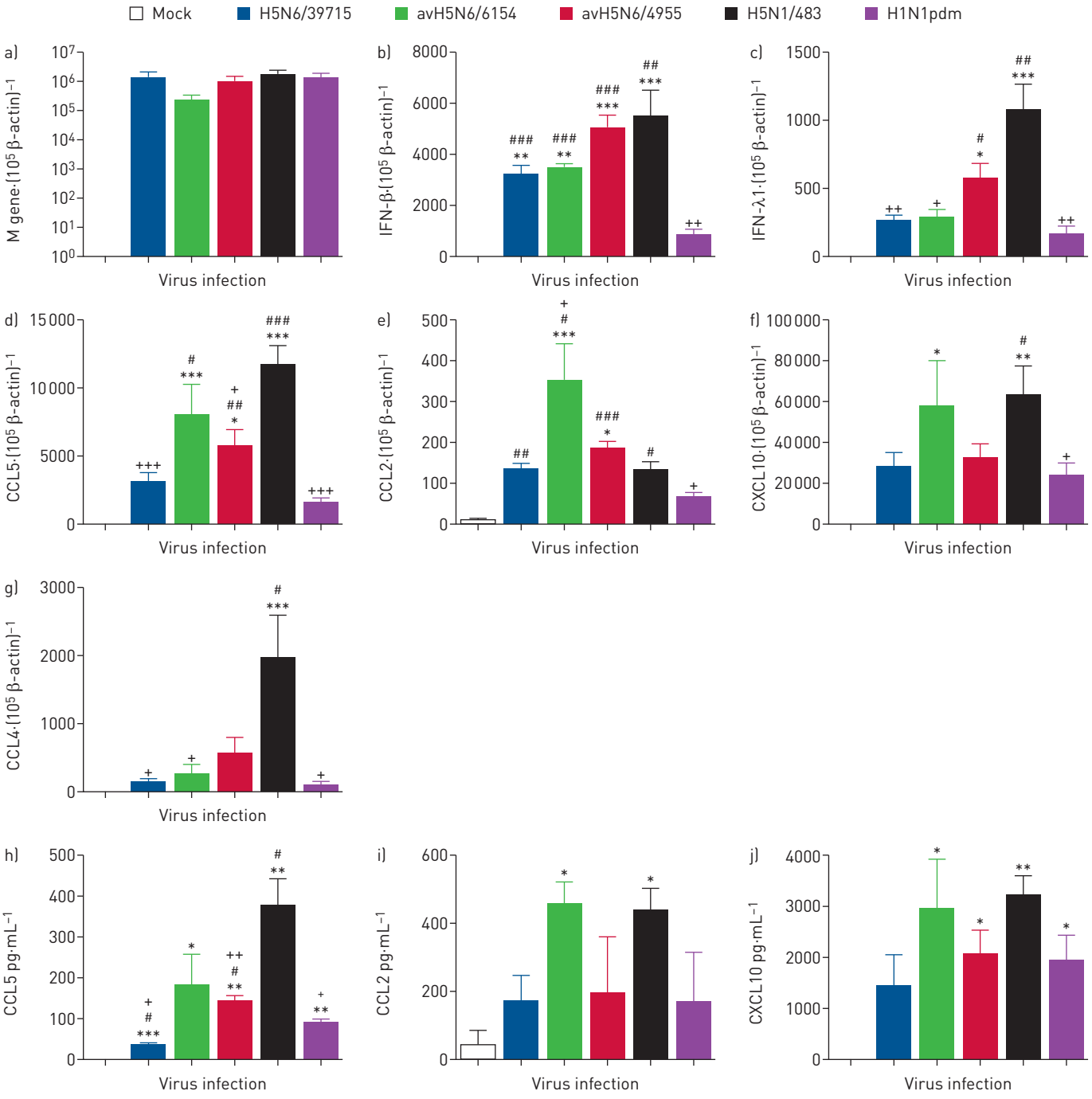


FIGURE 6 Cytokine and chemokine expression profiles in human alveolar epithelial cells (AECs) infected with influenza A viruses. The expression of mRNA (mRNA copies per 10^5 β-actin copies) for the influenza matrix (M) gene (a), interferon-β (IFN-β; b), interferon-λ1 (IFN-λ1; c), CC chemokine ligand 5 (CCL5; d), CC chemokine ligand 2 (CCL2; e), CXC chemokine 10 (CXCL10; f) and CC chemokine ligand 4 (CCL4; g) is shown for various viruses at 24 h post-infection. Data is pooled from three independent experiments and presented as mean±SEM. Protein concentration in culture supernatants for CCL5 (h), CCL2 (i) and CXCL10 (j) were measured at 24 h post-infection by cytometric bead array (CBA) immunoassay. The mean concentrations, pooled from three independent experiments, are shown and data are presented as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001 (compared to mock); #: p<0.05; ##: p<0.01; ###: p<0.001 (compared to H1N1pdm); +: p<0.05; ++: p<0.01; +++: p<0.001 (compared to H5N1/483). Statistical significance was calculated using Student's t-test.

H5N6 virus, the two avian H5N6 isolates replicated to titres similar to H5N1 but lower than H1N1pdm in *ex vivo* cultures of both human bronchus and lung. All three H5N6 viruses showed similar cellular tropism in *ex vivo* cultures of human bronchus and lung. The replication competence of these viruses in AECs was of a similar pattern to that seen in *ex vivo* lung cultures, except that for H1N1pdm which was lower, a

finding consistent with a previous report [20] in which titres of H1N1pdm increased until 24 h post-infection and then levelled-off afterwards.

The human H5N6 isolate replicated to the highest titres in AECs, while avH5N6/6154 and avH5N6/4955 replicated to titres similar to or lower than HPAI H5N1/483, respectively. The immunofluorescent staining of viral NP and M proteins also demonstrated the extensive levels of viral protein in the cytoplasm following human H5N6 infection, while the viral protein of the two bird isolates predominantly appeared in the nucleus.

The PB2 E627K amino-acid substitution is a well-recognized mammalian adaptation marker and is crucial for enhancing the replication efficiency and virulence of avian influenza viruses in mammals [21–23]. The higher replication competence of the human H5N6 isolate may be explained by the presence of the 627K residue in PB2 protein [11], as also seen in the H5N1/483 isolate, whereas the avH5N6 isolates contained the 627E residue in the PB2 protein. High replication competence in *ex vivo* cultures of human bronchus, together with the ability to infect *ex vivo* cultures of human nasopharyngeal tissue, suggest that the H5N6/39715-like viruses may potentially transmit from human-to-human. The two avian H5N6 viruses in this study possess 627E residues in PB2 protein, implying that their polymerase activity is not well adapted for optimal activity in human cells. It has been reported that serial passage of an avian H5N6 virus containing PB2 627E in mice leads to a change of residue (E-to-K) at position 627 [24] and this greatly enhances polymerase activity and virus replication in mammalian cells [21, 25–28]. This finding suggests that avian H5N6 viruses with a PB2 627E marker could acquire the mammalian adaptation mutation 627K. Indeed, it is possible that the avian virus that originally infected the patient from which the sample was obtained may have had a PB2 627E marker and the human virus we isolated, which had a PB2 627K marker, may have been the result of just such an adaptation taking place in the patient.

The human H5N6 isolate showed a preference for binding avian-like α-2,3-SA receptors, whereas both avian H5N6 isolates bound both avian-like α-2,3-SA receptors and human-like α-2,6-SA receptors. Both H5N1 viruses and the human H5N6 virus had a putative glycosylation site at HA amino acid residues 158–160, which was lost in the two avian H5N6 viruses. This may account for the difference in receptor-binding profile observed for SA-linked glycans.

The T160A substitution seen in the HA protein of the avian H5N6 isolates has been reported to be responsible for a shift in the binding properties of H5N1 viruses from avian-like to human-like receptors in guinea pigs [29]. This loss of a potential N-linked glycosylation site at residues 158–160 contributed to transmission of H5N1 viruses between guinea pigs by direct contact [29]. H5N6 isolates obtained from four waterfowl and live-poultry markets in China between 2013 and 2014 were found to have the T160A adaptation in their HA protein and to have binding affinity to human-like α-2,6-glycans [30, 31]. The avian H5N6 viruses bind paraffin-embedded human lung tracheal cells and AECs, and transmit by direct contact in ferrets but not by aerosol [30]. Efficient direct transmission of avian H5N6 viruses in ducks has also been reported [32]. There were no other known mammalian adaptation markers in residues 225–228 of the three H5N6 viruses that may contribute to these observed differences in receptor binding profiles. Based on antigenicity analysis, the current vaccines may fail to protect poultry against the H5 reassortants including H5N6 [32].

While many patients with H5N6 disease have a severe clinical course associated with severe pneumonia, multiple organ dysfunction and acute respiratory distress syndrome [7, 9, 10], sometimes associated with dissemination to extra-pulmonary organs [9], some patients did undergo milder illness [33]. The detection of cytokines and chemokines at both the mRNA and protein levels showed that while the H5N6 viruses we tested were not as high inducers of pro-inflammatory cytokines as H5N1, they were more potent inducers of these cytokines than was H1N1pdm. More H5N6 isolates have to be investigated to confirm these findings. Although the human H5N6/39715 virus did not elicit high levels of cytokines and chemokines in primary human AECs, high replication competence in the *ex vivo* cultures of human bronchus and lung can also lead to vast damage of the tissues.

From available epidemiological observations, it appears that the predominant route of acquiring H5N6 infection is *via* direct contact with infected poultry. However, our findings demonstrate that H5N6/39715 replicated efficiently in human upper and lower respiratory tract derived *ex vivo* cultures at a level comparable with H1N1pdm and was more efficient than HPAI H5N1/483. This suggests that these H5N6 viruses may have a greater capacity for poultry-to-human transmission and, possibly, human-to-human transmission than H5N1 viruses. Our findings, that the human H5N6 virus (which has α-2,3-SA affinity) replicated in the tissues of the upper airway, were in line with our previous observations that the HPAI H5N1 viruses (also having α-2,3-SA affinity) replicate in the human upper airway albeit with poorer replication competence than the human H5N6 virus [34]. The presence of the 627K residue in PB2 protein, although not solely sufficient, is a prerequisite for efficient person-to-person transmission [35]. By comparing the H5N6 gene sequences available so far, it has been noted that several human H5N6 isolates (except H5N6/39715) contain the T160A mutation in their HA proteins. The human H5N6 viruses A/Yunnan/0127/2015 (GISAID accession number:

EPI641398–EPI641404), A/Yunnan/14563/2015 (GISAID accession number: EPI587614–EPI587621) and A/Yunnan/14564/2015 (GISAID accession number: EPI587622–EPI587629), the last two being isolated from a 44-year-old male patient who succumbed to the disease, possess both HA T160A and PB2 E627K substitutions that are expected to have enhanced binding affinity to human-like α-2,6-SA and increased virulence and replication capacity, respectively. These observations suggest that human H5N6 isolates have the potential to evolve and gain mutations with improved human adaptation, perhaps even airborne transmissibility. H5N6 viruses can be isolated from air sampled from live-poultry markets in China [36] emphasising the need to implement control measures to minimise the spread H5N6 infection in poultry.

Human infection with avian influenza A/H7N9 viruses also continues to cause zoonotic disease with 803 laboratory-confirmed cases and 315 deaths reported so far. Influenza A/H7N9 viruses share a similar tissue tropism with the human H5N6 isolate investigated in this study as both viruses replicate well in *ex vivo* cultures of human bronchus and infect both ciliated and non-ciliated cells in the bronchial epithelium [16]. In human AEC cultures *in vitro*, both H5N6 and H7N9 were found to be less potent inducers of pro-inflammatory cytokines and chemokines when compared with HPAI H5N1 viruses [16, 36].

One limitation of our research was the relatively small number of H5N6 viruses studied. Furthermore, *ex vivo* cultures of the human respiratory tract are only surrogates for human beings. However, given the impossibility of carrying out experimental infections in human volunteers because of ethical concerns, the experimental model we have used here (where virus tropism and replication competence can be assessed in human bronchus, lung and nasopharynx) is probably the closest approximation to experimental human infection that is feasible. Thus, these data are likely to be more relevant to assessment of H5N6 viruses for zoonotic and pandemic risk than receptor binding studies using glycan arrays, since with *ex vivo* cultures we are investigating virus binding to physiologically relevant receptors found in the human respiratory tract and the subsequent steps of virus entry and replication.

Summary

In conclusion, our findings on the tropism and replication competence of H5N6 viruses in *ex vivo* human respiratory tract explant cultures suggest that the novel human influenza H5N6 viruses are better adapted to the human respiratory tract than the HPAI H5N1 viruses. The avian H5N6 isolates we studied have dual binding affinity to human-like receptors (α-2,6-glycans) as well as avian-like receptors (α-2,3-glycans). The high prevalence of H5N6 viruses in poultry in East and South East Asia poses a public health threat [31, 37, 38] and our results highlight the need for active surveillance in poultry, wild birds and humans who are in close contact with these sources. The novel H5N6 viruses are thus a cause of significant public health concern.

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References

1 Xu X, Subbarao, Cox NJ, *et al.* Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 1999; 261: 15–19.
2 World Health Organization. Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO, 2003-2016. www.who.int/influenza/human_animal_interface/2016_07_19_tableH5N1.pdf?ua=1 Date last accessed: February 21, 2017. Date last updated: July 19, 2016.
3 Zhao K, Gu M, Zhong L, *et al.* Characterization of three H5N5 and one H5N8 highly pathogenic avian influenza viruses in China. *Vet Microbiol* 2013; 163: 351–357.
4 Zhao G, Gu X, Lu X, *et al.* Novel reassortant highly pathogenic H5N2 avian influenza viruses in poultry in China. *PLoS One* 2012; 7: e46183.
5 World Organisation for Animal health (OIE). Update on highly pathogenic avian influenza in animals (type H5 and H7). www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2016/ Date last accessed: February 21, 2017. Date last updated: February 16, 2017.
6 Shen H, Wu B, Chen Y, *et al.* Influenza A (H5N6) virus reassortant, Southern China, 2014. *Emerg Infect Dis* 2015; 21: 1261–1262.
7 Pan M, Gao R, Lv Q, *et al.* Human infection with a novel, highly pathogenic avian influenza A (H5N6) virus: virological and clinical findings. *J Infect* 2016; 72: 52–59.
8 Yang ZF, Mok CK, Peiris JS, *et al.* Human infection with a novel avian influenza A (H5N6) virus. *N Engl J Med* 2015; 373: 487–489.

9 Gao R, Pan M, Li X, *et al.* Post-mortem findings in a patient with avian influenza A (H5N6) virus infection. *Clin Microbiol Infect* 2016; 22: 574.e1–574.e5.
10 Xu W, Li H, Jiang L. Human infection with a highly pathogenic avian influenza A (H5N6) virus in Yunnan province, China. *Infect Dis* 2016; 48: 477–482.
11 Mok CK, Da Guan W, Liu XQ, *et al.* Genetic characterization of highly pathogenic avian influenza A (H5N6) virus, Guangdong, China. *Emerg Infect Dis* 2015; 21: 2268–2271.
12 Steinhauer DA. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* 1999; 258: 1–20.
13 Stevens J, Blixt O, Tumpey TM, *et al.* Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science* 2006; 312: 404–410.
14 Hui KP, Li HS, Cheung MC, *et al.* Highly pathogenic avian influenza H5N1 virus delays apoptotic responses via activation of STAT3. *Sci Rep* 2016; 6: 28593.
15 Chan RW, Hemida MG, Kayali G, *et al.* Tropism and replication of Middle East Respiratory Syndrome coronavirus from dromedary camels in the human respiratory tract: an in-vitro and ex-vivo study. *Lancet Respir Med* 2014; 2: 813–822.
16 Chan MC, Chan RW, Chan LL, *et al.* Tropism and innate host responses of a novel avian influenza A H7N9 virus: an analysis of ex-vivo and in-vitro cultures of the human respiratory tract. *Lancet Respir Med* 2013; 1: 534–542.
17 Chan MC, Cheung CY, Chui WH, *et al.* Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir Res* 2005; 6: 135.
18 Chan MC, Chan RW, Yu WC, *et al.* Influenza H5N1 virus infection of polarized human alveolar epithelial cells and lung microvascular endothelial cells. *Respir Res* 2009; 10: 102.
19 Suzuki Y, Matsunaga M, Matsumoto M. N-Acetylneuraminyllactosylceramide, GM3-NeuAc, a new influenza A virus receptor which mediates the adsorption-fusion process of viral infection. Binding specificity of influenza virus A/Aichi/2/68 (H3N2) to membrane-associated GM3 with different molecular species of sialic acid. *J Biol Chem* 1985; 260: 1362–1365.
20 Chan MC, Chan RW, Yu WC, *et al.* Tropism and innate host responses of the 2009 pandemic H1N1 influenza virus in ex vivo and in vitro cultures of human conjunctiva and respiratory tract. *Am J Pathol* 2010; 176: 1828–1840.
21 Mehle A, Doudna JA. An inhibitory activity in human cells restricts the function of an avian-like influenza virus polymerase. *Cell Host Microbe* 2008; 4: 111–122.
22 Mehle A, Doudna JA. Adaptive strategies of the influenza virus polymerase for replication in humans. *Proc Natl Acad Sci USA* 2009; 106: 21312–21316.
23 Mok KP, Wong CH, Cheung CY, *et al.* Viral genetic determinants of H5N1 influenza viruses that contribute to cytokine dysregulation. *J Infect Dis* 2009; 200: 1104–1112.
24 Peng X, Wu H, Peng X, *et al.* Amino acid substitutions occurring during adaptation of an emergent H5N6 avian influenza virus to mammals. *Arch Virol* 2016; 161: 1665–1670.
25 Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J Virol* 1993; 67: 1761–1764.
26 Hatta M, Gao P, Halfmann P, *et al.* Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 2001; 293: 1840–1842.
27 Shinya K, Hamm S, Hatta M, *et al.* PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. *Virology* 2004; 320: 258–266.
28 Labadie K, Dos Santos Afonso E, Rameix-Welti MA, *et al.* Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. *Virology* 2007; 362: 271–282.
29 Gao Y, Zhang Y, Shinya K, *et al.* Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. *PLoS Pathog* 2009; 5: e1000709.
30 Sun H, Pu J, Wei Y, *et al.* Highly pathogenic avian influenza H5N6 viruses exhibit enhanced affinity for human type sialic acid receptor and in-contact transmission in model ferrets. *J Virol* 2016; 90: 6235–6243.
31 Bi Y, Zhang Z, Liu W, *et al.* Highly pathogenic avian influenza A (H5N1) virus struck migratory birds in China in 2015. *Sci Rep* 2015; 5: 12986.
32 Sun H, Pu J, Hu J, *et al.* Characterization of clade 2.3.4.4 highly pathogenic H5 avian influenza viruses in ducks and chickens. *Vet Microbiol* 2016; 182: 116–122.
33 Zhang R, Chen T, Ou X, *et al.* Clinical, epidemiological and virological characteristics of the first detected human case of avian influenza A (H5N6) virus. *Infect Genet Evol* 2016; 40: 236–242.
34 Nicholls JM, Chan MC, Chan WY, *et al.* Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. *Nat Med* 2007; 13: 147–149.
35 Hatta M, Hatta Y, Kim JH, *et al.* Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog* 2007; 3: 1374–1379.
36 Chan LL, Bui CT, Mok CK, *et al.* Evaluation of the human adaptation of influenza A/H7N9 virus in PB2 protein using human and swine respiratory tract explant cultures. *Sci Rep* 2016; 6: 35401.
37 Chen LJ, Lin XD, Guo WP, *et al.* Diversity and evolution of avian influenza viruses in live poultry markets, free-range poultry and wild wetland birds in China. *J Gen Virol* 2016; 97: 844–854.
38 Wong FY, Phommachanh P, Kalpravidh W, *et al.* Reassortant highly pathogenic influenza A (H5N6) virus in Laos. *Emerg Infect Dis* 2015; 21: 511–516.