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Characterization of substitutions in the neuraminidase of A(H7N9) influenza viruses selected following serial passage in the presence of different neuraminidase inhibitors

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ABSTRACT

Avian A(H7N9) infections in humans have been reported in China since 2013 and are of public health concern due to their severity and pandemic potential. Oseltamivir and peramivir are neuraminidase inhibitors (NAIs) routinely used for the treatment of A(H7N9) infections, but variants with reduced sensitivity to these drugs can emerge in patients during treatment. Zanamivir and laninamivir are NAIs that are used less frequently. Herein, we performed *in vitro* serial passaging experiments with recombinant viruses, containing the neuraminidase (NA) from influenza A/Anhui/1/13 (H7N9) virus, in the presence of each NAI, to determine whether variants with reduced sensitivity would emerge. NA substitutions were characterized for their effect on the NA enzymatic activity and surface expression of the A/Anhui/1/13 (Anhui/1) NA, as well as NAs originating from contemporary A(H7N9) viruses of the Yangtze River Delta and Pearl River Delta lineages. *In vitro* passage in the presence of oseltamivir, peramivir and laninamivir selected for substitutions associated with reduced sensitivity (E119D, R292K and R152K), whereas passage in the presence of zanamivir did not select for any viruses with reduced sensitivity. All the NA substitutions significantly reduced activity, but not the expression of the Anhui/1 NA. In contemporary N9 NAs, all substitutions tested significantly reduced NA enzyme function in the Yangtze River lineage background, but not in the Pearl River Delta lineage background. Overall, these findings suggest that zanamivir may be less likely than the other NAIs to select for resistance in A(H7N9) viruses and that the impact of substitutions that reduce NAI susceptibility or enzyme function may be less in A(H7N9) viruses from the Pearl River lineage.

1. Introduction

On March 31st, 2013, China alerted global public health officials of three human cases of infection with avian influenza A(H7N9) viruses originating from poultry (CDC, 2013). Since then there have been six annual epidemic waves of A(H7N9) human infections, with the fifth wave (2016–2017) characterized by increased genetic divergence amongst the viruses, leading to their separation into two antigenically distinct lineages - the Pearl River Delta and the Yangtze River Delta lineages and the emergence of highly-pathogenic (HPAI) A(H7N9) viruses (Imai et al., 2017; Zhang et al., 2015; Zhu et al., 2017). Only a small number of human infections with an HPAI A(H7N9) virus has been reported and while disease severity progressed rapidly in these patients, a comparison of the clinical impact of HPAI A(H7N9) virus infections compared to those with a LPAI A(H7N9) virus is limited due to the small sample size (Zhou et al., 2017b; Zhu et al., 2017). Influenza A(H7N9) infections in humans have been characterized by acute respiratory disease symptoms leading to pneumonia in 88% of the cases from the first four waves and a 36% case-fatality rate (Gao et al., 2013b; Iuliano et al., 2017). Most A(H7N9) infections result from close contact with poultry and while limited human-to-human transmission has been reported, there is no evidence to date of sustained transmission of the virus between humans (Li et al., 2014b; Xie et al., 2017; Zhou et al., 2017a).

Specific influenza A(H7N9) vaccines are not widely available for use in humans, although some countries are preparing stockpiles for

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pandemic preparedness (Isakova-Sivak and Rudenko, 2017). In the absence of an A(H7N9) vaccine, antivirals play an important role in the treatment of infected individuals and prophylaxis of healthcare workers in close contact with infected cases. Although two classes of influenza antivirals, the M2 ion channel inhibitors (adamantanes) and the neuraminidase inhibitors (NAIs), are widely available in China and other parts of the world, the S31N substitution in the M2 protein of all A(H7N9) viruses reported to date means that the adamantanes are not effective (Cao et al., 2014; Dong et al., 2015). Of the three NAIs that are licensed in China (oseltamivir, peramivir and zanamivir), most A(H7N9) infected patients are treated with either oseltamivir and/or peramivir (ChinaBioToday, 2013; Gao et al., 2013a; Kuehn, 2013; Li et al., 2014a; Xie et al., 2017). A small number of A(H7N9) patients have been treated with intravenous zanamivir (Ho et al., 2014; Yang et al., 2015), but laninamivir, now the most widely used NAI in Japan for the treatment of seasonal influenza, is not yet licensed in China and therefore has not been used as a treatment of A(H7N9) infection (Zaraket and Saito, 2016).

Although NAI treatment has been shown to result in clinical benefit in A(H7N9) patients (Hu et al., 2013b), the propensity for oseltamivir or peramivir treatment to select for A(H7N9) viruses with reduced sensitivity to NAIs, especially in severely ill patients requiring prolonged dosing, was identified early in the first wave of infections (Dong et al., 2018; Hai et al., 2013; Hu et al., 2013a; Ke et al., 2017; Lin et al., 2014; Yen et al., 2013). The most commonly selected variant viruses contained the NA amino acid substitution R292K (N2 numbering will be used throughout), which resulted in highly reduced inhibition by oseltamivir and peramivir, and mildly reduced inhibition by zanamivir (Dong et al., 2018; Hai et al., 2013; Hu et al., 2013a; Ke et al., 2017; Lin et al., 2014; Yen et al., 2013). Viruses bearing other NA substitutions (e.g. H274Y, E119V, I222K or I222R) have also been detected in A(H7N9) patients following oseltamivir or peramivir treatment and have been shown to reduce NAI susceptibility (Dong et al., 2018; Hai et al., 2013; Hu et al., 2013a; Li et al., 2014a).

Zanamivir and laninamivir share a greater structural similarity to sialic acid, the natural substrate of NA, than oseltamivir and peramivir (Ferraris and Lina, 2008; Gubareva, 2004). This similarity is hypothesized to be the reason that fewer zanamivir- or laninamivir-resistant seasonal influenza viruses are detected, compared to oseltamivir- or peramivir-resistant viruses (Gubareva et al., 2017a; Hurt et al., 2016; Meijer et al., 2014; Takashita et al., 2015). However, given the limited clinical experience with the use of zanamivir or laninamivir to treat A(H7N9) virus infections, it is not known whether they are also less likely to select for resistance in A(H7N9) viruses compared to oseltamivir or peramivir. We conducted serial passage of recombinant viruses containing the NA of A/Anhui/1/13 (H7N9) virus in the presence of increasing concentrations of each NAI, with the aim of determining the propensity of different NAIs to select for variants with reduced NAI sensitivity.

It is known that amino acid substitutions that reduce NAI sensitivity can also reduce viral fitness (Ferraris and Lina, 2008), and therefore for each amino acid substitution that altered NAI sensitivity, we further evaluated its impact on NA enzyme function. Since it is also known that NA substitutions can have a differential impact on enzyme function in different NA backgrounds (Bloom et al., 2010; Farrukee et al., 2015; Ferraris and Lina, 2008), the effect of specific substitutions was evaluated in the N9 NA derived from A/Anhui/1/2013 virus, as well as NAs derived from recent A(H7N9) viruses of the Yangtze River and Pearl River Delta lineages.

2. Materials and method

2.1. Cells

293T human embryonic kidney (HEK) cells and Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's Modified Eagle Medium (Gibco™, US) supplemented with 10% (v/v) fetal bovine serum (JRH Biosciences, US), 2 mM L-glutamine (SAFC Biosciences, US), 200 U/mL penicillin (Sigma-Aldrich, US), 200 µg/mL streptomycin (Sigma-Aldrich), 0.02 M HEPES (SAFC Biosciences) and 2 mg/L Amphotericin B (Fungizone) (Sigma Aldrich), and at 37 °C with 5% CO_2.

2.2. Generation of reverse genetic viruses and serial passaging

Viral RNA was extracted from stocks of the A/Anhui/1/2013 (Anhui/1) virus, kindly provided by the Chinese Center for Disease Control and Prevention. The NA gene for the Anhui/1 virus was amplified using the SuperScript® One-Step RT-PCR System for Long Templates kit (Life Technologies, US), using primers described in Hoffmann et al. (2001). The amplified NA was then incorporated into the pHW2000 reverse genetics (rg) plasmid (kindly provided by Dr. Richard Webby, St Jude Children's Research Hospital, Memphis, USA). The Anhui/1 NA plasmid was transfected together with seven pHW2000 plasmids containing the remaining influenza segments (PB2, PB1, PA, NP, HA, M, NS) derived from either A/PR/8/34 (PR8) or A/WSN/33 (WSN) virus, into co-cultures of 293T and MDCK cells, as described previously (Hoffmann et al., 2000). The resulting viruses were designated N9 + PR8 and N9 + WSN respectively.

Serial passaging of the recombinant viruses was performed in the presence of oseltamivir carboxylate, zanamivir, laninamivir or peramivir (Carbosynth, UK). MDCK cells were grown to confluence in T-25 flasks and infected with a low multiplicity of infection (MOI) of 0.01, as previously described (Hurt et al., 2010). Viruses were passaged ten times in MDCK cells in NAI concentrations that increased five-fold at each passage (such that the concentration ranged from 1 nM to 1953 µM across 10 passages). As a control, viruses were passaged in parallel in the absence of drug. Viral RNA was extracted from the supernatant after each passage using the QIAamp viral RNA mini kit and the NA and the haemagglutinin (HA) gene were sequenced as described previously (Hurt et al., 2010).

2.3. Clonal analysis of viral mixtures

A triple substitution was observed in a reverse genetics virus after the final passage, and to further investigate this, the NA gene was amplified from viral RNA using the SuperScript® One-Step RT-PCR System for Long Templates kit (Life Technologies, US). The resulting PCR product was cloned into the pCR-Blunt II-TOPO vector utilising the Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen, US) and transformed into One Shot™ TOP10 Chemically Competent *E. coli* cells (Invitrogen, US). The following day, 104 individual clones were randomly picked from the agar plates and further analysed by sequencing.

2.4. Mutagenesis and NAI susceptibility on expressed NA proteins

To evaluate the effect of each NA amino acid substitution on NAI susceptibility, wild-type and variant NA proteins were expressed in cell-culture and NAI susceptibility assays were performed.

The Anhui/1 NA gene was cloned into an expression plasmid with a V5 epitope tag. The plasmid, kindly provided by Dr. Jesse Bloom (Fred Hutchinson Cancer Research Center, Washington, USA) consists of a CMV promoter, 5′ EcoR1/3′Not I cloning sites, followed by an internal ribosome entry site (IRES) and expresses the green fluorescence protein (eGFP). Previous studies have established that an epitope tag at the end of the NA gene does not affect protein expression (Bloom et al., 2010, 2011). For the purposes of our studies, the plasmid was modified by replacing the EcoR1 restriction site with an *Eco*RV restriction site.

Site-directed mutagenesis was performed with the GeneArt Site-Directed mutagenesis kit (Thermofisher Scientific) and custom designed primers were used to introduce point mutations into the cloned Anhui/1 NA gene. The NA genes from a Yangtze River Delta lineage (HPAI A/Taiwan/1/2017 (GISAID ID: EPI_ISL_248778)) and a Pearl River Delta

lineage (LPAI A/Hong Kong/214/2017 (GISAID ID: EPI_ISL_242275)) virus were synthesized by Bioneer Ltd. (Melbourne, Australia). These genes were also incorporated into the expression plasmid and substitutions of interest inserted by site-directed mutagenesis. As the Yangtze-NA (A/Taiwan/1/17) naturally contained the R292K substitution which is known to alter NAI susceptibility, this substitution was removed prior to site-directed mutagenesis.

The NA proteins were then expressed by transfection of 293T cells as described previously (Bloom, 2014; Bloom et al., 2010, 2011; Butler et al., 2014; Farrukee et al., 2018). The cells were trypsinized 20-h post transfection and re-suspended in an assay buffer consisting of 15 mM MOPS, 145 mM sodium chloride, 2.7 mM potassium chloride, and 4.0 mM calcium chloride, adjusted to pH 7.4. Immediately before use, the assay buffer was supplemented with 2% heat-inactivated fetal bovine serum. The 293T cells, now expressing variant NA proteins, were then used in an in an NA inhibition assay performed with a fluorometric MUNANA (2-(4-methylumbelliferyl)-a-d-N-acetylneuraminic acid)-substrate as per standard protocol with viruses (Hurt et al., 2012), using oseltamivir carboxylate (oseltamivir), zanamivir, peramivir, and laninamivir to determine IC_{50} values (50% inhibitory concentration). Each assay was carried out in at least three separate replicates; however when required up to six separate replicates were done. The difference in NAI susceptibility of variants from WT was evaluated for significance using an unpaired two-tailed students t-test.

2.5. Neuraminidase surface expression and activity in different N9 backgrounds

Measurement of cell-surface NA expression and activity was also performed by transfecting 293T cells with an expression plasmid for 20 h as described in the previous section. Post-transfection, the cells were harvested, counted and assessed for NA activity and cell-surface expression. The NA enzyme activity on the cell surface was measured using a modified MUNANA-based assay (Bloom et al., 2011). The cell surface expression of the NA enzyme was measured by staining the cells with an Anti-V5 Alexaflour 647 antibody (Thermofisher, Australia) and staining intensity was determined using flow cytometry with gating on APC and FITC channels (Bloom et al., 2011). The GFP signal was used to control for transfection efficiency and the NA activity results were normalized to total surface expression. Three independent experiments were performed to assess NA expression and activity, where each variant NA was tested in triplicate. GraphPad Prism v.6 was used for two-way ANOVA analysis with Bonferroni's post-hoc analysis for between-group comparisons on the corrected fluorescence intensity (for NA activity) or APC staining intensity (for NA expression) between variant and wild-type NAs.

3. Results

3.1. Serial passage of reassortant N9 viruses in the presence of NAI

Reassortant viruses containing the NA gene of the Anhui/1 virus (N9 + PR8 and N9 + WSN) were passaged in the presence of increasing concentrations of zanamivir, oseltamivir, peramivir or laninamivir. In the absence of NAI pressure, the viruses were stable with no HA or NA amino acid substitutions arising following 10 passages (Table 1).

The R292K NA substitution was observed in a mixed population (as determined by double peaks in sequence chromatogram) in the N9 + PR8 virus after the 10th passage (P10) in the presence of oseltamivir (1953 μM), but no substitutions were detected in the N9 + WSN virus passaged under the same conditions (Table 1). A combination of substitutions R292K, P331S and E119D were observed at P10 (1953 μM) in the N9 + WSN virus after passaging in the presence of peramivir. To further evaluate if the triple substitutions R292K, P331S and E119D arose in combinations with each other in a single virus, the extracted viral RNA was cloned and 104 colonies were sequenced. The results show that the incidence of R292K + P331S + E119D (n = 1), R292K + E119D (n = 7), E119D + P331S (n = 7) and R292K + P331S (n = 3) in a single virus was lower than the frequency of single substitutions (n = 12–29) (Supplementary Table 1).

The R152K substitution was observed in a mixed population after P6 (3125 nM) in the N9 + PR8 virus passaged in peramivir. Laninamivir also selected for the R152K substitution in the N9 + PR8 virus from P8 (78 μM) onwards, but this NAI did not select for any substitutions in the N9 + WSN virus. No NA substitutions were detected in the N9 + WSN virus following passage in zanamivir, but passage of N9 + PR8 virus in zanamivir resulted in the detection of an R387K substitution as a mixed population at P3 (25 nM) that became fixed after P8 (78 μM).

HA amino acid substitutions were also seen in some of the recombinant viruses (Table 1).

3.2. NAI sensitivity, NA activity and surface expression of Anhui/1 N9 variants

The impact of each substitution observed in the Anhui/1 NA on NAI susceptibility was further investigated in an IC_{50} assay using expressed NA proteins. Substitution R292K in NA, which was selected *in vitro* following passage in the presence of oseltamivir or peramivir, led to significant susceptibility changes, with a > 1000-fold increase in oseltamivir and peramivir IC_{50} and a 41-fold & 21-fold increase in zanamivir and laninamivir IC_{50} respectively, compared to wild-type (WT) NA (Table 2). The E119D substitution, which was selected in the presence of peramivir, significantly increased zanamivir (412-fold), peramivir (222-fold), laninamivir (170-fold) and oseltamivir (4-fold)IC_{50} values. The R152K substitution, selected by passage in both peramivir and laninamivir, was associated with a 22-fold increase in laninamivir IC_{50} and 5-fold increase in zanamivir IC_{50}, which was significant. The R125K substitutions also increased peramivir IC_{50} by 8-fold, but this was not significant. The R387K and P331S substitutions are distant from the NA active site (Figure S1) and were both shown to not affect NAI susceptibility (Table 2).

To further understand the impact of each NA substitution on the enzymatic properties of the Anhui/1 NA protein, we used an *in vitro* plasmid expression assay in 293T cells. Compared to the WT NA, all NA substitutions examined were associated with significantly reduced NA activity, with the substitutions R292K and E119D reducing enzymatic activity by the greatest amount (> 90% relative to WT NA) (Fig. 1). Compared to WT, the R387K, R152K and P331S NA substitutions reduced NA activity by 38%, 56% and 73%, respectively. The E119D, R387K and P331S substitutions also significantly reduced relative NA expression.

3.3. Evaluation of relevant NA amino acid substitutions in contemporary N9 NAs

To determine if the findings observed in the Anhui/1 NA would extend to NAs from contemporary A(H7N9) viruses, we introduced the three NA substitutions that altered NAI susceptibility (E119D, R292K and R152K) into the NA from a Yangtze River Delta lineage (Yangtze) virus and a Pearl River Delta lineage (Pearl) virus. A phylogenetic tree showing the evolutionary relationship between these viruses is visualized in figure S2.

The effect of these substitutions on NAI susceptibility was measured in the Yangzte-NA and Pearl-NA background. The results in Table 2 show that in the Pearl-NA background, similar to the Anhui/1 NA background, R292K leads to a greater than 1000-fold increase in oseltamivir and peramivir IC_{50}, and the E119D substitution conferred the greatest changes to zanamivir (752-fold) and peramivir (499-fold) IC_{50}. Similarly, the R152K substitution lead to significant increases in zanamivir (8-fold) and laninamivir (32-fold) IC_{50}s. The effect of the E119D substitution in the Yangzte-NA background could not be measured due to low NA activity. The IC_{50} changes in the Yangtze-NA background due

Table 1
Summary of NA substitution arising after serial passaging of N9 reassortant viruses in increasing NAI drug concentrations.

Passage no.	Drug Conc.	Zanamivir		Oseltamivir		Peramivir		Laninamivir		No Drug	
		N9 + WSN	N9 + PR8	N9 + WSN	N9 + PR8	N9 + WSN	N9 + PR8	N9 + WSN	N9 + PR8	N9 + WSN	N9 + PR8
1	1 nM	–	–	–	–	–	–	–	–	–	–
2	5 nM	–	–	–	–	–	–	–	–	–	–
3	25 nM	–	R387 R/K	–	–	–	–	–	–	–	–
4	125 nM	–	R387 R/K	–	–	–	–	–	–	–	–
5	625 nM	–	R387 R/K	–	–	–	–	–	–	–	–
6	3125 nM	–	R387 R/K	–	–	–	R152 R/K	–	–	–	–
7	16 μM	–	R387 R/K	- [b]	-[a]	-[c]	R152 R/K	-[c]	–	–	–
8	78 μM	–	R387K[a]	- [b]	-[a]	-[c]	R152K	-[c]	R152 R/K	–	–
9	391 μM	–	R387K[a]	- [b]	-[a]	-[c]	R152K	-[c]	R152K	–	–
10	1953 μM	–	R387K[a]	- [b]	R292 R/K[a]	E119E/D[c] R292 R/K P331 P/S	R152 R/K	-[c]	R152 R/K	–	–

"–" No substitution in NA observed.
[a] HA substitution A240T was observed, HA numbering starting from first methionine.
[b] HA substitution S151L was observed, HA numbering starting from first methionine.
[c] HA substitution S185G was observed, HA numbering starting from first methionine.

to the R292K and R152K substitution followed similar trends to those observed in Anhui/1 NA and Pearl-NA, although the fold increase in laninamivir IC_{50} due to R152K was somewhat lower (5-fold as opposed to 22 or 32-fold).

In the context of NA enzymatic activity, the three NA substitutions in the Yangtze-NA had a similar impact to those observed in the Anhui/1 NA. Comparatively, in the Pearl-NA, substitutions E119D or R292K showed lower reductions in NA activity and the R152K substitution had a higher NA activity compared to the WT (Fig. 2). The E119D substitution caused significantly reduced NA expression in both the Pearl and Yangtze-NA backgrounds, as it did in the Anhui/1 NA, but the R292K and R152K substitutions only caused a significant reduction in NA expression in the Yangtze-NA, and not in the Pearl-NA background. Of note, the WT Yangtze-NA had 1.6-fold higher activity and 2.1-fold higher expression than the WT Pearl-NA (data not shown).

In addition to the three NA substitutions detected in this study that altered NAI susceptibility, we evaluated a further five other NA substitutions from A(H7N9) viruses that have previously been reported to alter NAI susceptibility (E119V, I222T, I222R, I222K, and H274Y) (Dong et al., 2018; Kiso et al., 2017; Marjuki et al., 2015a, 2015b) (Supplementary Figure 1). The impact of these substitutions on reducing NAI susceptibility was further confirmed in this study (Table 2). As expected all substitutions tested led to significant increases in IC_{50} values to at least one NAI tested, in both N9 backgrounds.

All substitutions significantly reduced relative NA activity in the Yangtze-NA background but only E119D, R292K and H274Y

Table 2
Summary of changes in NAI susceptibility due to substitutions in different N9 backgrounds.

Mean IC_{50} (nM) ± SD (fold-difference)[#]				
NA substitution	Zanamivir	Oseltamivir	Peramivir	laninamivir
Anhui/1 NA				
WT	1.4 ± 0.8	0.7 ± 0.3	0.1 ± 0.0	0.9 ± 0.2
E119D	578.8 ± 299.7 (412)*	3.1 ± 2.1 (4)*	22.2 ± 10.2 (222)*	152.9 ± 85.9 (170)*
R292K	58.1 ± 29.7 (41)*	16934.4 ± 7605 (24191)*	161.3 ± 47.9 (1613)*	18.8 ± 6.6 (21)*
R152K	6.6 ± 1.3 (5)*	0.4 ± 0.2 (1)	0.8 ± 0.4 (8)	20.2 ± 7.3 (22)*
P331S	1.3 ± 1.0 (1)	0.3 ± 0.2 (1)	0.3 ± 0.3 (3)	0.9 ± 0.2 (1)
R387K	1.3 ± 0.7 (1)	0.4 ± 0.2 (1)	0.4 ± 0.5 (4)	0.7 ± 0.3 (1)
Pearl-NA				
WT	0.7 ± 0.3	0.7 ± 0.4	0.1 ± 0.1	0.4 ± 0.2
E119D	526.5 ± 122.0 (752)*	8.1 ± 3.7 (12)*	49.9 ± 11.6 (499)*	103.1 ± 29.4 (258)*
R292K	50.0 ± 23.6 (71)*	13646.1 ± 4471.3 (19494)*	320.1 ± 98.8 (3201)*	16.0 ± 4.9 (40)*
R152K	5.2 ± 1.6 (8)*	0.8 ± 0.3 (1)	1.4 ± 1.1 (14)*	12.8 ± 5.3 (32)*
H274Y	2.4 ± 1.2 (3)*	42.8 ± 0.3 (61)*	1.8 ± 1.3 (18)*	2.3 ± 1.3 (6)*
I222T	4.9 ± 2.5 (7)*	13.3 ± 7.8 (19)*	1.2 ± 0.8 (12)*	3.2 ± 2.1 (8)*
I222R	6.3 ± 3.6 (9)	28.0 ± 8.1 (40)*	1.1 ± 0.4 (11)*	4.6 ± 1.7 (12)*
I222K	3.3 ± 1.1 (5)*	12.4 ± 6.4 (17)*	0.7 ± 0.4 (7)	3.7 ± 2.7 (9)
E119V	10.5 ± 4.7 (15)*	271.5 ± 33.5 (388)*	0.8 ± 0.4 (8)*	1.1 ± 0.6 (3)
Yangtze-NA				
WT	1.3 ± 0.2	2.4 ± 0.87	0.2 ± 0.0	1.8 ± 0.1
E119D	–	–	–	–
R292K	47.2 ± 20.7 (36)*	8421.3 ± 1563.9 (3508)*	182.1 ± 35.7 (910)*	14.4 ± 5.9 (8)*
R152K	2.7 ± 0.5 (1)	0.9 ± 0.5 (0.4)	1.0 ± 0.7 (5)	8.6 ± 1.2 (5)*
H274Y	1.6 ± 0.3 (1)	140.1 ± 22.3 (58) *	2.2 ± 0.4 (11)*	2.2 ± 0.5 (1)
I222T	–	–	–	–
I222R	–	–	–	–
I222K	5.4 ± 1.8 (4.1)*	37.8 ± 12.2 (16)*	0.7 ± .02 (4)*	6.7 ± 2.1 (3.7)*
E119V	3.5 ± 1.8 (3)	324.4 ± 31.3 (135)*	0.5 ± 0.1 (3)*	1.6 ± 0.3 (1)

[#] All fold-differences are calculated based on IC_{50} wild-type values (first row).
* Indicates significant difference in IC_{50} value from WT.
'-' NA activity too low to measure IC_{50}.

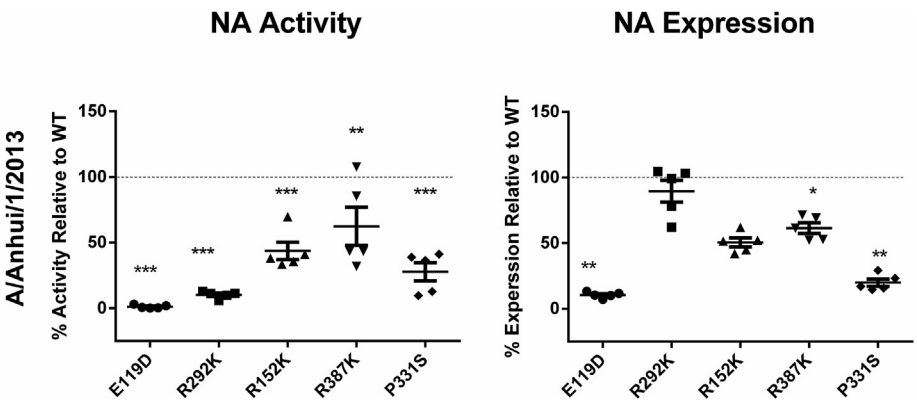

Fig. 1. Relative cell-surface NA activity and expression following introduction of NA substitutions observed during serial passaging of reassortant viruses expressing the Anhui/1 NA *in vitro*. The Anhui/1 NA gene with substitutions was cloned into an expression plasmid and transfected into 293T cells. 20 h post-transfection cells expressing NA proteins were harvested and measured for cell-surface NA enzymatic activity (fluorescence-based) and NA expression (FACS). Three independent experiments were performed, where each variant NA was tested in triplicate. Two-way ANOVA analysis with Bonferroni's post-hoc analysis was performed for comparison of the corrected fluorescence intensity (for NA activity) or the APC staining intensity (for NA expression) between the variant and wild-type NAs. *p < 0.05, **p < 0.01, ***p < 0.001.

significantly reduced relative NA activity in the Pearl-NA (Fig. 2). A similar trend was seen in relative NA expression where, with the exception of H274Y, all substitutions reduced relative NA expression to a greater extent in the Yangtze-NA background than in the Pearl-NA background. The biggest difference was seen with the I222T substitution, which impaired enzyme function completely in the Yangtze-NA background but only reduced NA activity and NA expression by 20% in the Pearl-NA background.

4. Discussion

This study used reassortant viruses (containing the NA of the A/Anhui/1/2013 A(H7N9) virus on a PR8 or WSN backbone) in serial passaging experiments to identify amino acid substitutions that may emerge under antiviral pressure with different NAIs. The impact of NA amino acid substitutions (detected in our passaging experiments or reported to impact NAI sensitivity in other studies) was evaluated for their effect on NA activity and expression. The selection of the R292K NA substitution under oseltamivir and peramivir pressure *in vitro* correlated with clinical observations, where A(H7N9) viruses with this NA substitution have been detected in patients during treatment with oseltamivir or peramivir (Dong et al., 2018; Hai et al., 2013; Hu et al., 2013a; Ke et al., 2017; Lin et al., 2014; Yen et al., 2013). The N9 NA from other avian influenza viruses (A/Tern/Australia/G70c/75 (H1N9) or A/Duck/Memphis/546/1974 (H11N9)) has also previously been shown to develop the R292K NA substitution following *in vitro* passage in the presence of oseltamivir (Molla et al., 2002; Song et al., 2015).

In this study, serial passage in the presence of peramivir selected for the E119D substitution in the N9 + PR8 virus, which led to highly reduced inhibition by zanamivir, peramivir and laninamivir in all N9 backgrounds. Previous *in vitro* work on recombinant variant NA proteins by Gubareva et al. has also shown E119D to confer highly reduced inhibition by zanamivir, laninamivir and peramivir, with similar fold-increases to our studies (Gubareva et al., 2017b). To our knowledge, the E119D substitution in A(H7N9) viruses has not been reported in clinical settings but an alternative substitution of E to V at position 119 has been described, although this change only alters sensitivity to oseltamivir (Marjuki et al., 2015b). Our results also show that the R152K substitution, which has been reported previously to arise in influenza B viruses following zanamivir treatment (Gubareva et al., 1998), can emerge in A(H7N9) viruses under peramivir or laninamivir pressure. However, while this substitution leads to highly reduced zanamivir inhibition (1000-fold) in influenza B viruses, the IC_{50} changes observed in the N9 viruses were less drastic, a finding in accordance with a

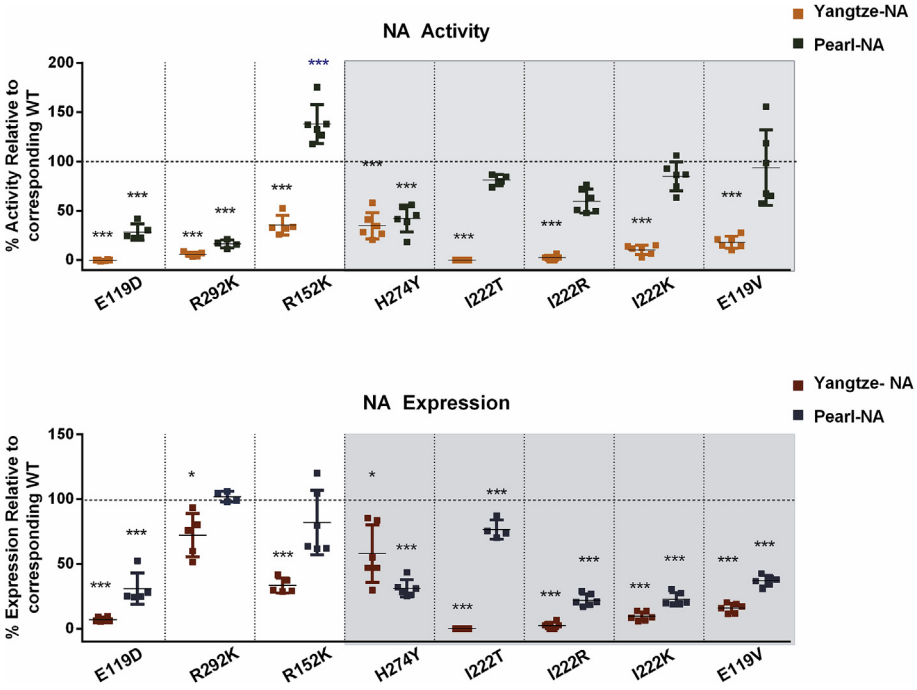

Fig. 2. Changes in relative cell-surface NA activity and expression due to substitutions in the Yangtze-NA and Pearl-NA background. NA genes were synthesized, mutated and cloned into an expression plasmid for NA expression and activity analysis. Three independent experiments were done to assess NA expression and activity, where each variant NA was tested in triplicate. We examined NA substitutions detected following serial passage of reassortant viruses in the presence of different NAIs (white background), as well as substitutions reported in other studies (grey background). Two-way ANOVA analysis with Bonferroni's post-hoc analysis was performed for comparisons of the corrected fluorescence intensity (for NA activity) or the APC staining intensity (for NA expression) between the variant and wild-type NAs. *p < 0.05, **p < 0.01, ***p < 0.001.

previous study (Gubareva et al., 2017a), confirming that the effect of a NA substitution on NAI susceptibility may vary depending on virus type or subtype (Abed et al., 2006; Farrukee et al., 2015).

In this study a triple substitution of R292K, E119D and P331S was initially observed in the in N9 + WSN virus after 10 passages in the presence of peramivir. This raised the possibility that P331S may be compensatory for R292K or E119D. However further analysis showed that the E119D and R292K substitutions occurred more frequently as single substitutions as opposed to double or triple substitution combinations in this virus. This information alongside the observation that P331S significantly reduced NA activity and expression compared to wild-type, suggests to us that P331S is unlikely to be compensatory.

It was of interest that no amino acid substitutions associated with reduced inhibition were observed in the N9 NA following passage in the presence of zanamivir. This may indicate that selection of A(H7N9) NAI-resistant variants following zanamivir treatment is less likely in a clinical setting than with oseltamivir or peramivir. Zanamivir resistance is less commonly observed in seasonal influenza viruses than oseltamivir resistance, and this is thought to be because of its closer structural similarity with sialic acid when compared to oseltamivir (Gubareva et al., 2017a; Hurt et al., 2016; Itzstein et al., 1993; Meijer et al., 2014; Takashita et al., 2015), but may also be associated with less use of the drug to treat influenza infections in general.

This study also showed that the in Anhui/1 NA, the R292K substitution significantly reduced NA activity relative to WT NA but had no significant effect on NA expression. Previous *in vitro* work with the A/Shanghai/1/2013 (H7N9) virus bearing the R292K substitution showed that even though this substitution reduced NA enzyme activity and substrate affinity, variants expressing this substitution replicated to similar titres as those of wild-type viruses in normal human bronchial epithelial and MDCK cells (Hai et al., 2013; Yen et al., 2013). However, studies in mice and ferrets showed that A(H7N9) viruses with the R292K substitution are less pathogenic and replicate to lower titres than wild-type viruses in both the upper respiratory tract and lungs (Imai et al., 2017; Marjuki et al., 2015b; Zhang et al., 2014). This suggests that whilst reduced NA activity may not alter *in vitro* replication, it can impact *in vivo* replication, although the exact mechanisms underlying these differences are not clear.

It is known that genetic differences of the NA gene within the same subtype can lead to a differential impact on viral fitness when particular NA substitutions are present (Abed et al., 2011, 2014; Baz et al., 2010; Bloom et al., 2010; Butler et al., 2014). This was clearly observed in our study, where many of the NA substitutions significantly reduced relative NA activity in the Yangtze-NA but not in the Pearl-NA. Previous studies with seasonal A(H1N1) viruses showed that within the same viral subtype, the H274Y substitution reduced enzyme activity significantly in older viruses like A/Caledonia/20/99 or A/Solomon Island/03/2006 but not in more recent A/Brisbane/59-2007-like viruses (Abed et al., 2011, 2014; Baz et al., 2010; Bloom et al., 2010; Butler et al., 2014). Structural and genetic analysis of the Pearl-NA, which differs from the Yangtze-NA by 19 amino acids, may reveal the possible causes behind the differential outcomes observed in this study, which would inform about the extent of A(H7N9) viruses with a NA background that may be more permissive to NA substitutions that confer NAI resistance. Finally, it should be noted that while the *in vitro* NA surface expression/activity experiments performed in this study can be useful for an initial assessment of viral fitness (Bloom et al., 2010, 2011; Butler et al., 2014), these results may not necessarily translate to improved replication or transmissibility *in vivo*.

The use of N9 + WSN or N9 + PR8 reassortant viruses for serial passaging experiments represents a limitation of our study, as the results do not account for the role of the HA and other A(H7N9) internal genes that may contribute to the emergence of variants with reduced NAI sensitivity. However, the use of reassortant viruses and an *in vitro* analysis was necessary in this study to reduce the bio-safety risks associated with generating A(H7N9) viruses with reduced NAI susceptibility.

Imbalance in the NA/HA of the reassortant viruses likely contributed to the HA substitutions observed in our experiments, and it is not clear if similar HA substitutions would have emerged if viruses expressing the matched HA genes were used. Previous studies have shown that HAsubstitutions can arise alongside NA substitutions, either as part of cell-culture adaptation or to restore NA/HA balance, during passaging experiments (Barnett et al., 1999; Blick et al., 1998; Gubareva et al., 1996, 1997; McKimm-Breschkin et al., 1996, 1998; Tai et al., 1998). In our study, substitutions S158G and S151L arose in the WSN HA and A240T arose in the PR8 HA during serial passaging. It is likely that the S151L and A240T substitutions are cell culture adaptations, as changes in equivalent residues in H3 and H5 HAs have been shown to be adaptive to human alpha 2,6 sialic acids (Busch et al., 2008; Gambaryan et al., 2006; Naughtin et al., 2011). Interestingly an HA amino acid substitution S158L was observed in another study, where reassortant N9 viruses with the A/NWS/33 backbone were serially passaged in increasing oseltamivir concentrations (Barnett et al., 1999; Blick et al., 1998; Gubareva et al., 1996, 1997; McKimm-Breschkin et al., 1996, 1998; Molla et al., 2002; Tai et al., 1998). The amino acid S158 (S145 in H3 numbering) is positioned at the HA antigenic site and substitutions at equivalent positions in H9 HA were shown to increase virulence in mice (Kaverin et al., 2004, 2007). Further study on the role of HA substitution S158G in viral fitness could be of great interest.

Overall, our study shows that particular substitutions associated with reduced sensitivity to NAI were selected *in vitro* by passaging reassortant viruses bearing the N9 NA in the presences of oseltamivir, peramivir and laninamivir, but not zanamivir. Selection of R292K variants in the presence of oseltamivir and peramivir in this study and in clinical settings is of concern. From a clinical perspective, using zanamivir may reduce the risk of emergence of R292K variants. This study also shows that many substitutions associated with reduced sensitivity to NAI were less debilitating to the enzyme function of the NA from a Pearl River Delta-like virus, relative to that of a Yangtze River Delta-like virus. While further *in vitro* and *in vivo* experiments are needed to understand the implication of this finding, it remains prudent to monitor A(H7N9) viruses from the Pearl River Delta lineage for NA substitutions associated with reduced NAI sensitivity prior to antiviral therapy.

Declaration of interest

No conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.05.009.

References

Abed, Y., Baz, M., Boivin, G., 2006. Impact of neuraminidase mutations conferring influenza resistance to neuraminidase inhibitors in the N1 and N2 genetic backgrounds. Antivir. Ther. 11, 971–976.

Abed, Y., Pizzorno, A., Bouhy, X., Boivin, G., 2011. Role of permissive neuraminidase mutations in influenza A/Brisbane/59/2007-like (H1N1) viruses. PLoS Pathog. 7, 8.

Abed, Y., Pizzorno, A., Bouhy, X., Rheaume, C., Boivin, G., 2014. Impact of potential permissive neuraminidase mutations on viral fitness of the H275Y oseltamivir-resistant influenza A(H1N1)pdm09 virus in vitro, in mice and in ferrets. J. Virol. 88, 1652–1658.

Barnett, J.M., Cadman, A., Burrell, F.M., Madar, S.H., Lewis, A.P., Tisdale, M., Bethell, R., 1999. In vitro selection and characterisation of influenza B/Beijing/1/87 isolates

with altered susceptibility to zanamivir. Virology 265, 286–295.
Baz, M., Abed, Y., Simon, P., Hamelin, M.E., Boivin, G., 2010. Effect of the neuraminidase mutation H274Y conferring resistance to oseltamivir on the replicative capacity and virulence of old and recent human influenza A(H1N1) viruses. J. Infect. Dis. 201, 740–745.
Blick, T.J., Sahasrabudhe, A., McDonald, M., Owens, I.J., Morley, P.J., Fenton, R.J., McKimm-Breschkin, J.L., 1998. The Interaction of Neuraminidase and Hemagglutinin Mutations in Influenza Virus in Resistance to 4-Guanidino-Neu5Ac2en. Virology 246, 95–103.
Bloom, J.D., 2014. An experimentally determined evolutionary model dramatically improves phylogenetic fit. Mol. Biol. Evol. 31, 1956–1978.
Bloom, J.D., Gong, L.I., Baltimore, D., 2010. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science 328, 1272–1275.
Bloom, J.D., Nayak, J.S., Baltimore, D., 2011. A computational-experimental approach identifies mutations that enhance surface expression of an oseltamivir-resistant influenza neuraminidase. PLoS One 6, e22201.
Busch, M.G., Bateman, A.C., Landolt, G.A., Karasin, A.I., Brockman-Schneider, R.A., Gern, J.E., Suresh, M., Olsen, C.W., 2008. Identification of amino acids in the HA of H3 influenza viruses that determine infectivity levels in primary swine respiratory epithelial cells. Virus Res. 133, 269–279.
Butler, J., Hooper, K.A., Petrie, S., Lee, R., Maurer-Stroh, S., Reh, L., Guarnaccia, T., Baas, C., Xue, L., Vitesnik, S., Leang, S.K., McVernon, J., Kelso, A., Barr, I.G., McCaw, J.M., Bloom, J.D., Hurt, A.C., 2014. Estimating the fitness advantage conferred by permissive neuraminidase mutations in recent oseltamivir-resistant A(H1N1)pdm09 influenza viruses. PLoS Pathog. 10.
Cao, R.Y., Xiao, J.H., Cao, B., Li, S., Kumaki, Y., Zhong, W., 2014. Inhibition of novel reassortant avian influenza H7N9 virus infection in vitro with three antiviral drugs, oseltamivir, peramivir and favipiravir. Antivir. Chem. Chemother. 23, 237–240.
CDC, 2013. Emergence of avian influenza A(H7N9) virus causing severe human illness - China, February-April 2013. MMWR Morb. Mortal. Wkly. Rep. 62, 366–371.
ChinaBioToday, 2013. China's SFDA ready to fast-track approvals of peramivir, a flu treatment. http://www.chinabiotoday.com/articles/20130408_1.
Dong, G., Peng, C., Luo, J., Wang, C., Han, L., Wu, B., Ji, G., He, H., 2015. Adamantane-resistant influenza a viruses in the world (1902–2013): frequency and distribution of M2 gene mutations. PLoS One 10, e0119115.
Dong, Z., Xia, Y., Ya, X., Chen, L., Liu, C., Wang, R., Shen, Q., 2018. Epidemiological and Genetic Characteristics of the Fifth Avian Influenza A(H7N9) Wave in Suzhou, China, from October 2016 to April 2017. Virus Genes.
Farrukee, R., Leang, S.K., Butler, J., Lee, R.T., Maurer-Stroh, S., Tilmanis, D., Sullivan, S., Mosse, J., Barr, I.G., Hurt, A.C., 2015. Influenza viruses with B/Yamagata- and B/Victoria-like neuraminidases are differentially affected by mutations that alter antiviral susceptibility. J. Antimicrob. Chemother. 70, 2004–2012.
Farrukee, R., Zarebski, A.E., McCaw, J.M., Bloom, J.D., Reading, P.C., Hurt, A.C., 2018. Characterization of influenza B virus variants with reduced neuraminidase inhibitor susceptibility. Antimicrob. Agents Chemother. 62 01081-01018.
Ferraris, O., Lina, B., 2008. Mutations of neuraminidase implicated in neuraminidase inhibitors resistance. J. Clin. Virol. 41, 13–19.
Gambaryan, A., Tuzikov, A., Pazynina, G., Bovin, N., Balish, A., Klimov, A., 2006. Evolution of the receptor binding phenotype of influenza A (H5) viruses. Virology 344, 432–438.
Gao, H.N., Lu, H.Z., Cao, B., Du, B., Shang, H., Gan, J.H., 2013a. Clinical findings in 111 cases of influenza A (H7N9) virus infection. N. Engl. J. Med. 368.
Gao, H.N., Lu, H.Z., Cao, B., Du, B., Shang, H., Gan, J.H., Lu, S.H., Yang, Y.D., Fang, Q., Shen, Y.Z., Xi, X.M., Gu, Q., Zhou, X.M., Qu, H.P., Yan, Z., Li, F.M., Zhao, W., Gao, Z.C., Wang, G.F., Ruan, L.X., Wang, W.H., Ye, J., Cao, H.F., Li, X.W., Zhang, W.H., Fang, X.C., He, J., Liang, W.F., Xie, J., Zeng, M., Wu, X.Z., Li, J., Xia, Q., Jin, Z.C., Chen, Q., Tang, C., Zhang, Z.Y., Hou, B.M., Feng, Z.X., Sheng, J.F., Zhong, N.S., Li, L.J., 2013b. Clinical findings in 111 cases of influenza A (H7N9) virus infection. N. Engl. J. Med. 368, 2277–2285.
Gubareva, L.V., 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res. 103, 199–203.
Gubareva, L.V., Besselaar, T.G., Daniels, R.S., Fry, A., Gregory, V., Huang, W., Hurt, A.C., Jorquera, P.A., Lackenby, A., Leang, S.-K., Lo, J., Pereyaslov, D., Rebelo-de-Andrade, H., Siqueira, M.M., Takashita, E., Odagiri, T., Wang, D., Zhang, W., Meijer, A., 2017a. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2015–2016. Antivir. Res. 146, 12–20.
Gubareva, L.V., Bethell, R., Hart, G.J., Murti, K.G., Penn, C.R., Webster, R.G., 1996. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. J. Virol. 70, 1818–1827.
Gubareva, L.V., Matrosovich, M.N., Brenner, M.K., Bethell, R.C., Webster, R.G., 1998. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J. Infect. Dis. 178, 1257–1262.
Gubareva, L.V., Robinson, M.J., Bethell, R.C., Webster, R.G., 1997. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en. J. Virol. 71, 3385–3390.
Gubareva, L.V., Sleeman, K., Guo, Z., Yang, H., Hodges, E., Davis, C.T., Baranovich, T., Stevens, J., 2017b. Drug susceptibility evaluation of an influenza A(H7N9) virus by analyzing recombinant neuraminidase proteins. J. Infect. Dis. 216, S566–S574.
Hai, R., Schmolke, M., Leyva-Grado, V.H., Thangavel, R.R., Margine, I., Jaffe, E.L., Krammer, F., Solorzano, A., Garcia-Sastre, A., Palese, P., Bouvier, N.M., 2013. Influenza A(H7N9) virus gains neuraminidase inhibitor resistance without loss of in vivo virulence or transmissibility. Nat. Commun. 4.
Ho, P.-L., Sin, W.-C., Chan, J.F.-W., Cheng, V.C.-C., Chan, K.-H., 2014. Severe influenza A H7N9 pneumonia with rapid virological response to intravenous zanamivir. Eur. Respir. J. 44, 535–537.
Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G., 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A. 97, 6108–6113.
Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289.
Hu, Y., Lu, S., Song, Z., Wang, W., Hao, P., Li, J., 2013a. Association between Adverse Clinical Outcome in Human Disease Caused by Novel Influenza A H7N9 Virus and Sustained Viral Shedding and Emergence of Antiviral Resistance. Lancet (London, England). pp. 381.
Hu, Y., Lu, S., Song, Z., Wang, W., Hao, P., Li, J., Zhang, X., Yen, H.L., Shi, B., Li, T., Guan, W., Xu, L., Liu, Y., Wang, S., Tian, D., Zhu, Z., He, J., Huang, K., Chen, H., Zheng, L., Li, X., Ping, J., Kang, B., Xi, X., Zha, L., Li, Y., Zhang, Z., Peiris, M., Yuan, Z., 2013b. Association between adverse clinical outcome in human disease caused by novel influenza A H7N9 virus and sustained viral shedding and emergence of antiviral resistance. Lancet 381, 2273–2279.
Hurt, A.C., Besselaar, T.G., Daniels, R.S., Ermetal, B., Fry, A., Gubareva, L., Huang, W., Lackenby, A., Lee, R.T., Lo, J., Maurer-Stroh, S., Nguyen, H.T., Pereyaslov, D., Rebelo-de-Andrade, H., Siqueira, M.M., Takashita, E., Tashiro, M., Tilmanis, D., Wang, D., Zhang, W., Meijer, A., 2016. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2014-2015. Antivir. Res. 132, 178–185.
Hurt, A.C., Nor'e, S.S., McCaw, J.M., Fryer, H.R., Mosse, J., McLean, A.R., Barr, I.G., 2010. Assessing the viral fitness of oseltamivir-resistant influenza viruses in ferrets, using a competitive-mixtures model. J. Virol. 84, 9427–9438.
Hurt, A.C., Okomo-Adhiambo, M., Gubareva, L.V., 2012. The fluorescence neuraminidase inhibition assay: a functional method for detection of influenza virus resistance to the neuraminidase inhibitors. Methods Mol. Biol. 865, 115–125.
Imai, M., Watanabe, T., Kiso, M., Nakajima, N., Yamayoshi, S., Iwatsuki-Horimoto, K., Hatta, M., Yamada, S., Ito, M., Sakai-Tagawa, Y., Shirakura, M., Takashita, E., Fujisaki, S., McBride, R., Thompson, A.J., Takahashi, K., Maemura, T., Mitake, H., Chiba, S., Zhong, G., Fan, S., Oishi, K., Yasuhara, A., Takada, K., Nakao, T., Fukuyama, S., Yamashita, M., Lopes, T.J.S., Neumann, G., Odagiri, T., Watanabe, S., Shu, Y., Paulson, J.C., Hasegawa, H., Kawaoka, Y., 2017. A highly pathogenic avian H7N9 influenza virus isolated from A human is lethal in some ferrets infected via respiratory droplets. Cell Host Microbe 22, 615–626.
Isakova-Sivak, I., Rudenko, L., 2017. Tackling a novel lethal virus: a focus on H7N9 vaccine development. Expert Rev. Vaccines 16, 1–13.
Itzstein, M.v., Wen-Yang, W., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B., Van Phan, T., Smythe, M.L., White, H.F., Oliver, S.W., Colman, P.M., Varghese, J.N., Ryan, M.D., Woods, J.M., Bethell, R.C., Hotham, V.J., Cameron, J.M., Penn, C.R., 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature 418.
Iuliano, A.D., Jang, Y., Jones, J., Davis, C.T., Wentworth, D.E., Uyeki, T.M., Roguski, K., Thompson, M.G., Gubareva, L., Fry, A., Burns, E., Trock, S., Zhou, S., Katz, J.M., Jernigan, D.B., 2017. Increase in human infections with avian influenza A(H7N9) virus during the fifth epidemic — China, october 2016–february 2017. MMWR Morb. Mortal. Wkly. Rep. 66, 254–255.
Kaverin, N.V., Rudneva, I.A., Govorkova, E.A., Timofeeva, T.A., Shilov, A.A., Kochergin-Nikitsky, K.S., Krylov, P.S., Webster, R.G., 2007. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J. Virol. 81, 12911–12917.
Kaverin, N.V., Rudneva, I.A., Ilyushina, N.A., Lipatov, A.S., Krauss, S., Webster, R.G., 2004. Structural differences among hemagglutinins of influenza a virus subtypes are reflected in their antigenic architecture: analysis of H9 escape mutants. J. Virol. 78, 240–249.
Ke, C., Mok, C.K.P., Zhu, W., Zhou, H., He, J., Guan, W., Wu, J., Song, W., Wang, D., Liu, J., Lin, Q., Chu, D.K.W., Yang, L., Zhong, N., Yang, Z., Shu, Y., Peiris, J.S.M., 2017. Human infection with highly pathogenic avian influenza A(H7N9) virus, China. Emerg. Infect. Dis. 23, 1332–1340.
Kiso, M., Iwatsuki-Horimoto, K., Yamayoshi, S., Uraki, R., Ito, M., Nakajima, N., Yamada, S., Imai, M., Kawakami, E., Tomita, Y., Fukuyama, S., Itoh, Y., Ogasawara, K., Lopes, T.J.S., Watanabe, T., Moncla, L.H., Hasegawa, H., Friedrich, T.C., Neumann, G., Kawaoka, Y., 2017. Emergence of oseltamivir-resistant H7N9 influenza viruses in immunosuppressed cynomolgus macaques. J. Infect. Dis. 216, 582–593.
Kuehn, B.M., 2013 May 22. CDC: use antivirals early, aggressively for H7N9 flu. J. Am. Med. Assoc. 309 (20), 2086. https://doi.org/10.1001/jama.2013.6086.
Li, Q., Zhou, L., Zhou, M., Chen, Z., Li, F., Wu, H., 2014a. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N. Engl. J. Med. 370.
Li, Q., Zhou, L., Zhou, M., Chen, Z., Li, F., Wu, H., Xiang, N., Chen, E., Tang, F., Wang, D., Meng, L., Hong, Z., Tu, W., Cao, Y., Li, L., Ding, F., Liu, B., Wang, M., Xie, R., Gao, R., Li, X., Bai, T., Zou, S., He, J., Hu, J., Xu, Y., Chai, C., Wang, S., Gao, Y., Jin, L., Zhang, Y., Luo, H., Yu, H., Wang, X., Gao, L., Pang, X., Liu, G., Yan, Y., Yuan, H., Shu, Y., Yang, W., Wang, Y., Wu, F., Uyeki, T.M., Feng, Z., 2014b. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N. Engl. J. Med. 370, 520–532.
Lin, P.H., Chao, T.L., Kuo, S.W., Wang, J.T., Hung, C.C., Lin, H.C., Yang, Z.Y., Ho, S.Y., Chang, C.K., Huang, M.S., Chen, H.H., Chen, Y.C., Lai, H.S., Chang, S.Y., Chang, S.C., Yang, P.C., 2014. Virological, serological, and antiviral studies in an imported human case of avian influenza A(H7N9) virus in Taiwan. Clin. Infect. Dis. 58, 242–246.
Marjuki, H., Mishin, V.P., Chesnokov, A.P., De La Cruz, J.A., Davis, C.T., Villanueva, J.M., Fry, A.M., Gubareva, L.V., 2015a. Neuraminidase mutations conferring resistance to oseltamivir in influenza A(H7N9) viruses. J. Virol. 89, 5419–5426.
Marjuki, H., Mishin, V.P., Chesnokov, A.P., Jones, J., Cruz, J.A., Sleeman, K., 2015b. Characterization of drug-resistant influenza A(H7N9) variants isolated from an oseltamivir-treated patient in Taiwan. J. Infect. Dis. 211.
McKimm-Breschkin, J.L., Blick, T.J., Sahasrabudhe, A., Tiong, T., Marshall, D., Hart, G.J., Bethell, R.C., Penn, C.R., 1996. Generation and characterization of variants of NWS/

G70C influenza virus after in vitro passage in 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en. Antimicrob. Agents Chemother. 40, 40–46.
McKimm-Breschkin, J.L., Sahasrabudhe, A., Blick, T.J., McDonald, M., Colman, P.M., Hart, G.J., Bethell, R.C., Varghese, J.N., 1998. Mutations in a Conserved Residue in the Influenza Virus Neuraminidase Active Site Decreases Sensitivity to Neu5Ac2en-Derived Inhibitors. J. Virol. 72, 2456–2462.
Meijer, A., Rebelo-de-Andrade, H., Correia, V., Besselaar, T., Drager-Dayal, R., Fry, A., Gregory, V., Gubareva, L., Kageyama, T., Lackenby, A., Lo, J., Odagiri, T., Pereyaslov, D., Siqueira, M.M., Takashita, E., Tashiro, M., Wang, D., Wong, S., Zhang, W., Daniels, R.S., Hurt, A.C., 2014. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2012-2013. Antivir. Res. 110, 31–41.
Molla, A., Kati, W., Carrick, R., Steffy, K., Shi, Y., Montgomery, D., Gusick, N., Stoll, V.S., Stewart, K.D., Ng, T.I., Maring, C., Kempf, D.J., Kohlbrenner, W., 2002. In vitro selection and characterization of influenza a (a/N9) virus variants resistant to a novel neuraminidase inhibitor, A-315675. J. Virol. 76, 5380–5386.
Naughtin, M., Dyason, J.C., Mardy, S., Sorn, S., von Itzstein, M., Buchy, P., 2011. Neuraminidase inhibitor sensitivity and receptor-binding specificity of Cambodian clade 1 highly pathogenic H5N1 influenza virus. Antimicrob. Agents Chemother. 55, 2004–2010.
Song, M.-S., Marathe, B.M., Kumar, G., Wong, S.-S., Rubrum, A., Zanin, M., Choi, Y.-K., Webster, R.G., Govorkova, E.A., Webby, R.J., 2015. Unique determinants of neuraminidase inhibitor resistance among N3, N7, and N9 avian influenza viruses. J. Virol. 89, 10891–10900.
Tai, C.Y., Escarpe, P.A., Sidwell, R.W., Williams, M.A., Lew, W., Wu, H., Kim, C.U., Mendel, D.B., 1998. Characterization of human influenza virus variants selected in vitro in the presence of the neuraminidase inhibitor GS 4071. Antimicrob. Agents Chemother. 42, 3234–3241.
Takashita, E., Meijer, A., Lackenby, A., Gubareva, L., Rebelo-de-Andrade, H., Besselaar, T., Fry, A., Gregory, V., Leang, S.K., Huang, W., Lo, J., Pereyaslov, D., Siqueira, M.M., Wang, D., Mak, G.C., Zhang, W., Daniels, R.S., Hurt, A.C., Tashiro, M., 2015. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013-2014. Antivir. Res. 117, 27–38.
Xie, J., Weng, Y., Ou, J., Zhao, L., Zhang, Y., Wang, J., Chen, W., Huang, M., Xiu, W., Chen, H., Wu, B., He, W., Zhu, Y., You, L., Huang, Z., Zhang, C., Hong, L., Wang, W., Zheng, K., 2017. Epidemiological, clinical, and virologic features of two family clusters of avian influenza A (H7N9) virus infections in Southeast China. Sci. Rep. 7, 017–01761.
Yang, Z.F., Mok, C.K.P., Liu, X.Q., Li, X.B., He, J.F., Guan, W.D., Xu, Y.H., Pan, W.Q., Chen, L.Y., Lin, Y.P., Wu, S.G., Pan, S.H., Huang, J.C., Ding, G.Y., Zheng, K., Ke, C.W., Lin, J.Y., Zhang, Y.H., Lee, H.H.Y., Liu, W.K., Yang, C.G., Zhou, R., Peiris, J.S.M., Li, Y.M., Chen, R.C., Chen, L., Zhong, N.S., 2015. Clinical, virological and immunological features from patients infected with Re-emergent avian-origin human H7N9 influenza disease of varying severity in guangdong province. PLoS One 10, e0117846.
Yen, H.L., McKimm-Breschkin, J.L., Choy, K.T., Wong, D.D., Cheung, P.P., Zhou, J., Ng, I.H., Zhu, H., Webby, R.J., Guan, Y., Webster, R.G., Peiris, J.S., 2013. Resistance to neuraminidase inhibitors conferred by an R292K mutation in a human influenza virus H7N9 isolate can be masked by a mixed R/K viral population. mBio 4 00396-00313.
Zaraket, H., Saito, R., 2016. Japanese surveillance systems and treatment for influenza. Curr. Treat. Options Infect. Dis. 8, 311–328.
Zhang, X., Song, Z., He, J., Yen, H.L., Li, J., Zhu, Z., Tian, D., Wang, W., Xu, L., Guan, W., Liu, Y., Wang, S., Shi, B., Zhang, W., Qin, B., Cai, J., Wan, Y., Xu, C., Ren, X., Chen, H., Liu, L., Yang, Y., Zhou, X., Zhou, W., Xu, J., Peiris, M., Hu, Y., Yuan, Z., 2014. Drug susceptibility profile and pathogenicity of H7N9 influenza virus (Anhui1 lineage) with R292K substitution. Emerg. Microb. Infect. 3, 12.
Zhang, Y., Shen, Z., Ma, C., Jiang, C., Feng, C., Shankar, N., Yang, P., Sun, W., Wang, Q., 2015. Cluster of human infections with avian influenza a (H7N9) cases: a temporal and spatial analysis. Int. J. Environ. Res. Public Health 12, 816–828.
Zhou, L., Ren, R., Yang, L., Bao, C., Wu, J., Wang, D., Li, C., Xiang, N., Wang, Y., Li, D., Sui, H., Shu, Y., Feng, Z., Li, Q., Ni, D., 2017a. Sudden increase in human infection with avian influenza A(H7N9) virus in China, September-December 2016. Western Pac Surveill Response J 8, 6–14.
Zhou, L., Tan, Y., Kang, M., Liu, F., Ren, R., Wang, Y., Chen, T., Yang, Y., Li, C., Wu, J., Zhang, H., Li, D., Greene, C.M., Zhou, S., Iuliano, A.D., Havers, F., Ni, D., Wang, D., Feng, Z., Uyeki, T.M., Li, Q., 2017b. Preliminary epidemiology of human infections with highly pathogenic avian influenza A(H7N9) virus, China, 2017. Emerg. Infect. Dis. 23, 1355–1359.
Zhu, W., Zhou, J., Li, Z., Yang, L., Li, X., Huang, W., Zou, S., Chen, W., Wei, H., Tang, J., Liu, L., Dong, J., Wang, D., Shu, Y., 2017. Biological characterisation of the emerged highly pathogenic avian influenza (HPAI) A(H7N9) viruses in humans, in mainland China, 2016 to 2017. Euro Surveill. 22, 1560–7917.