Zanamivir-resistant influenza viruses with Q136K or Q136R neuraminidase residue mutations can arise during MDCK cell culture creating challenges for antiviral susceptibility monitoring

K Little [1 2] , **S Leang** [1] , **J Butler** [1] , **C Baas** [1 2] , **B Harrower** [3] , **J Mosse** [2] , **IG Barr** [1] , **AC Hurt** [1 4]
1. WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Victoria, Australia
2. Monash University, School of Applied Sciences and Engineering, Churchill, Victoria, Australia
3. Queensland Health Services, Coopers Plains, Queensland, Australia
4. Melbourne School of Population and Global Health, University of Melbourne, Victoria, Australia

Correspondence: Aeron Hurt (aeron.hurt@influenzacentre.org

Surveillance of circulating influenza strains for antiviral susceptibility is important to ensure patient treatment guidelines remain appropriate. Influenza A(H3N2) and A(H1N1)pdm09 virus isolates containing mutations at the Q136 residue of the neuraminidase (NA) that conferred reduced susceptibility to the NA inhibitor (NAI) zanamivir were detected during antiviral susceptibility monitoring. Interestingly, the mutations were not detectable in the viruses from respective clinical specimens, only in the cultured isolates. We showed that variant viruses containing the Q136K and Q136R NA mutations were preferentially selected in Madin-Darby canine kidney epithelial (MDCK) cells, but were less well supported in MDCK-SIAT1 cells and embryonated eggs. The effect of Q136K, Q136R, Q136H and Q136L substitutions in NA subtypes N1 and N2 on NAI susceptibility and in vitro viral fitness was assessed. This study highlights the challenges that cell culture derived mutations can pose to the NAI susceptibility analysis and interpretation and reaffirms the need to sequence viruses from respective clinical specimens to avoid misdiagnosis. However, we also demonstrate that NA mutations at residue Q136 can confer reduced zanamivir, peramivir or laninamivir susceptibility, and therefore close monitoring of viruses for mutations at this site from patients being treated with these antivirals is important.

Introduction

Neuraminidase inhibitors (NAIs) are a class of influenza antivirals that target the highly conserved enzymatic site of the neuraminidase (NA) glycoprotein on the surface of influenza A and B viruses [1]. The NAIs have become the most widely used antivirals for the tr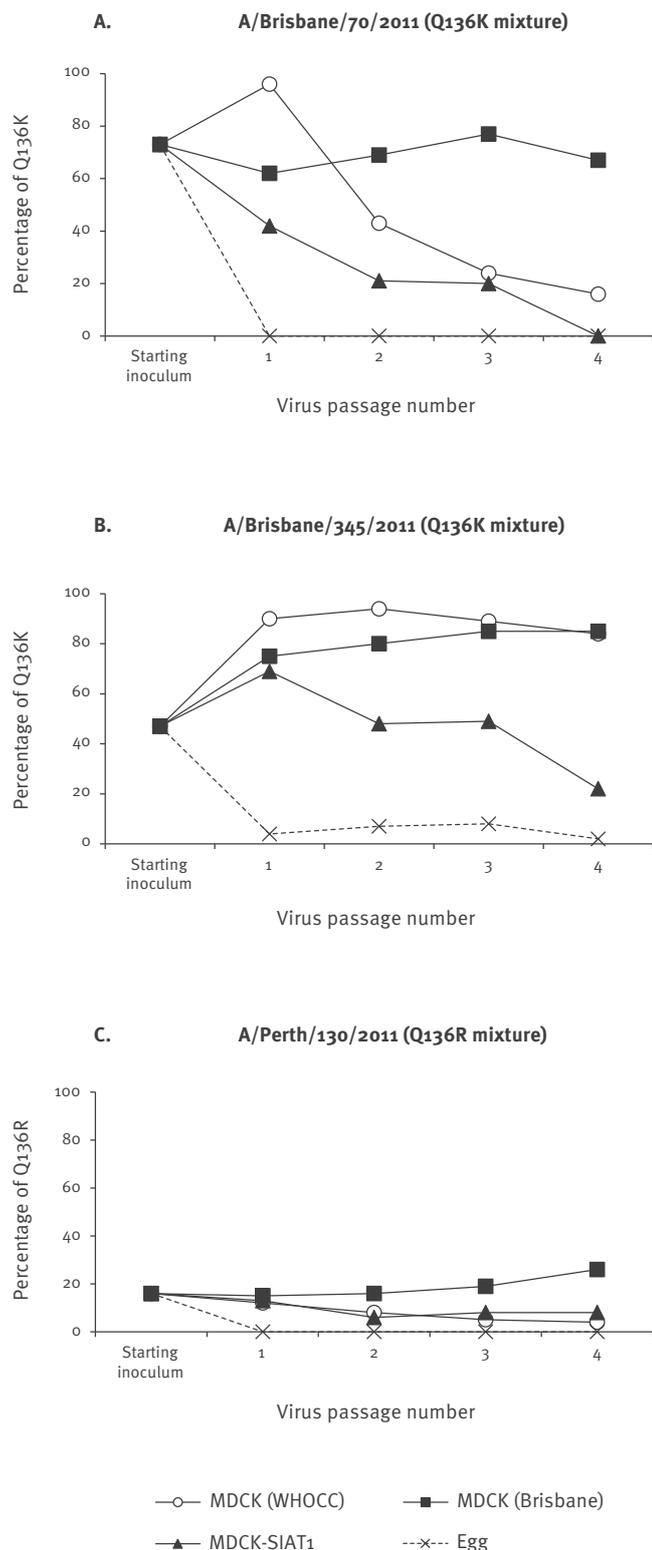eatment or prophylaxis of influenza, particularly since the development of widespread resistance to the adamantanes, the older class of antivirals that block the M2 ion channel protein [2]. Two NAIs, oseltamivir and zanamivir, have been available in many countries since 1999, and two new NAIs, peramivir and laninamivir, have recently been approved for human use in Japan and a small number of other countries. Each of the NAIs is structurally different and therefore binds slightly differently within the NA active site [1]. This difference in binding is advantageous for treatment, as a virus that develops resistance against one NAI, may retain sensitivity to others. For example, the H275Y NA mutation in NA subtype N1-containing viruses confers resistance to oseltamivir but not to zanamivir [3].

Resistance to the NAIs commonly occurs as a result of amino acid mutations within the NA active site, either in the catalytic residues (those that interact directly with the NAIs), or in the framework residues (those that provide structural support for the catalytic residues) [4]. However, not all viruses with resistance to NAIs will pose a public health risk, as mutations that reduce binding can also impact the ability of the NA to interact with the natural substrate during replication [5]. However, in some cases, the mutation can affect NAI sensitivity but not compromise viral 'fitness'. The H275Y mutation that was present in seasonal influenza A(H1N1) viruses between 2007 and 2009 is such an example, as it conferred oseltamivir resistance but did not appear to affect the ability of the virus to replicate and transmit [6,7].

Oseltamivir resistant influenza viruses have been detected at a considerably higher frequency than zanamivir resistant viruses. During human clinical trials,

Serial passage of A(H1N1)pdm09 influenza virus isolates (n=3) in different Madin-Darby canine kidney (MDCK) cells and embryonated hens' eggs

A.   A/Brisbane/70/2011 (Q136K mixture)

B.   A/Brisbane/345/2011 (Q136K mixture)

C.   A/Perth/130/2011 (Q136R mixture)

- ○ MDCK (WHOCC)
- ▲ MDCK-SIAT1
- ■ MDCK (Brisbane)
- --×-- Egg

WHO CC: World Health Organization Collaborating Centre for Reference and Research on Influenza.

Three isolates were used, two containing a mixture of wildtype (WT) and variant viruses with a Q136K neuraminidase (NA) mutation (panels A and B) and one containing a mixture of WT and variant viruses with a Q136R NA mutation (panel C). Proportion of Q136K or Q136R was determined by pyrosequencing analysis.

oseltamivir resistance was detected in ‹1–4% of adults [8,9] and 5–6% of treated children [10] undergoing oseltamivir treatment, although in observational studies the frequency of resistance in oseltamivir treated children has been as high as 27% [11]. Most significantly, oseltamivir-resistant seasonal A(H1N1) viruses with an H275Y mutation became widespread during 2008, spreading globally even in regions of low drug usage [12,13]. In comparison, there have only been a few reports of zanamivir resistance. The first was in an immunocompromised child undergoing zanamivir treatment where an influenza B virus with a R152K NA mutation was detected that caused a 40–150-fold reduction in zanamivir sensitivity [14,15]. More recently a small number of A(H1N1)pdm09 viruses with an I223R NA mutation have been detected in immunocompromised patients exposed to oseltamivir and/or zanamivir [16,17] and in a patient without previous exposure to NAIs [18], but the change in zanamivir sensitivity as a result of this mutation is relatively minor (10-fold), compared with the larger 45-fold shift in oseltamivir sensitivity [16].

Previously, our group and others reported the detection of former seasonal A(H1N1) virus isolates with a Q136K mutation that conferred a 250-fold reduction in zanamivir sensitivity [19,20]. The Q136K isolates were particularly unusual because the mutation could not be detected in the clinical specimens from which they were derived. This suggested that either the variant virus was being generated in cell culture or it was present in very low levels in the clinical specimen and then selectively amplified during cell culture. The former seasonal A(H1N1) virus stopped circulating soon after the emergence of the A(H1N1)pdm09 virus in 2009, and for the first two years after the A(H1N1)pdm09 viruses started circulating, no Q136K variants were reported. However, here we report the detection of both Q136K and Q136R substitutions in A(H1N1)pdm09 and A(H3N2) viruses between 2011 and 2014, investigate their selection in different cell lines and determine the effect that these and other amino substitutions of the Q136 residue have on NAI susceptibility and NA enzymatic function.

Methods

Virus strains, Madin-Darby canine kidney epithelial cells and virus culture

The influenza viruses used in this study were received at the World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza (WHOCC), Melbourne, Australia through the WHO Global Influenza Surveillance and Response System (GISRS) from countries in the Asia Pacific region. The Q136K or Q136R isolates had initially been isolated and then passaged in MDCK cells in external laboratories before being received and repassaged one to two further times at the WHOCC. WHOCC MDCK cells were originally received from ATCC (CCL-34) and used at passage level 63 to 83 and grown in Dulbecco's modified

FIGURE 2

Thermostability of influenza viruses expressing wildtype, Q136R, Q136K, Q136L or Q136H substituted neuraminidases

A. A(H1N1)pdm09

B. A(H3N2)
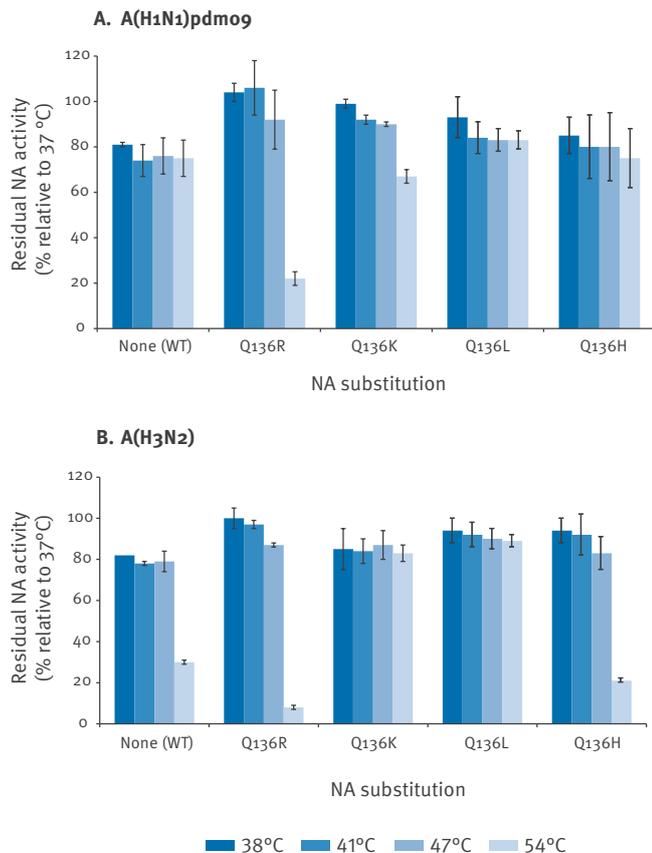
NA: neuraminidase; SD: standard deviation; WT: wildtype.

Residual activity is expressed as a percentage of activity relative to that at 37 °C. Mean values are presented and error bars indicate ± SD from three separate experiments.

Eagle's medium (DMEM)/Ham's F12/Coon's medium with L-glutamine (SAFC Biosciences) supplemented with 2 nM L-glutamine (SAFC Biosciences), 1X Eagle's minimum essential medium (MEM) non-essential amino acids (Sigma), 0.06% (v/v) sodium bicarbonate (Sigma), 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (Sigma), 2% penicillin–streptomycin solution (Sigma) and 10% fetal bovine serum (SAFC Biosciences). Viruses were cultured in MDCK cells in DMEM/Ham's F12/Coon's maintenance medium with L-glutamine (SAFC Biosciences) supplemented with 2 nM L-glutamine (SAFC Biosciences), 1X MEM non-essential amino acids (Sigma), 0.06% (v/v) sodium bicarbonate (Sigma), 0.02 M HEPES buffer solution (Sigma), 2% penicillin-streptomycin solution (Sigma), 2 µg/mL fungizone (amphotericin B) (SAFC Biosciences) and 4 µg/mL trypsin (Sigma).

Neuraminidase activity and neuraminidase inhibition assays

To determine NA activity, viruses were standardised to an equivalent haemagglutinin (HA) titre using turkey red blood cells, serially diluted (twofold) in assay buffer (32.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.5, 4 mM $CaCl_2$ with 0.1% nonyl phenoxypolyethoxylethanol (NP-40)), and then mixed with an equal volume (50 µL) of the fluorescence substrate 2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) (0.3 mM) (Sigma) before incubation at 37 °C for 60 min. The reaction was terminated by the addition of 100 µL of stop solution (0.14 M NaOH in 83% ethanol). Quantification of the fluorescent product 4-methylumbelliferone was determined using a Fluoroscan Ascent FL (Thermo) with an excitation wavelength of 360 nm and an emission wavelength of 448 nm. Viruses were tested in duplicate in three separate assays. The NA activity of the recombinant variant viruses was calculated as a relative percentage of the NA activity of the recombinant wildtype (WT) virus at the same virus dilution.

Viruses were tested for susceptibility to the NAIs laninamivir, oseltamivir carboxylate, peramivir, and zanamivir, which were kindly provided by Daiichi-Sankyo, Japan, Hoffman-La Roche Ltd, Switzerland, BioCryst Pharmaceuticals Inc., United States (US), and GlaxoSmithKline, United Kingdom (UK), respectively. To determine the drug concentration required to inhibit 50% of the NA activity (IC_{50}), 50 µL of virus, diluted according to the NA activity assay, was mixed with varying concentrations of inhibitor in microtitre plates (FluoroNunc plates, Nunc). Final reaction mixture concentrations of the NAIs ranged from 0.01 nM to 10,000 nM. The virus/NAI mix was incubated at room temperature for 45 min before the addition of 50 µL of MUNANA substrate (0.3 mM) and then incubated at 37 °C for 60 min. The reaction was terminated by addition of 100 µL of the stop solution. The data were plotted as the percentage of fluorescence activity inhibited against the log NAI concentration. IC_{50} values were calculated using the logistic curve fit programme 'Robosage' kindly provided by GlaxoSmithKline, UK.

Reverse transcription-polymerase chain reaction, sequencing and pyrosequencing

Reverse transcription-polymerase chain reaction (RT-PCR) was conducted using gene specific primers (sequences available on request) using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase Kit (Life Technologies) according to the manufacturer's instructions. Sequencing was conducted using the ABI Prism Dye Terminator III Cycle Sequencing Kit (Life Technologies) and analysed on the ABI 3500XL automated DNA sequencer (Life Technologies) and data aligned using the DNAStar Lasergene 8 software package. Pyrosequencing to determine the relative proportion of a Q136 variant in a viral population used the PyroMark ID System (QIAGEN). Briefly, 40 µL biotinylated PCR product was

Figure 3

Haemagglutinin/neuraminidase balance of influenza viruses expressing wildtype, Q136R, Q136K, Q136L or Q136H neuraminidases following incubation at 4°C, 37°C and in the absence of epithelial cells (control)

A. A(H1N1)pdm09

B. A(H3N2)

■ Control ■ 4°C ■ 37°C
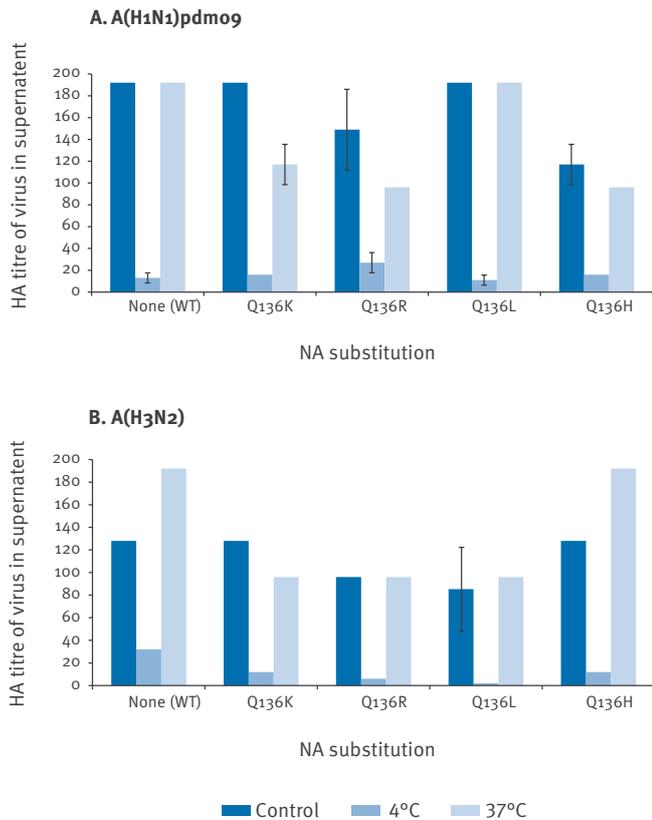
HA: haemagglutinin; NA: neuraminidase; SD: standard deviation; WT: wildtype.

Mean values are presented and error bars indicate ± SD from three separate experiments. Where error bars are not visible, the same HA titre was obtained across the three experiments.

immobilised on streptavidin-coated sepharose beads (QIAGEN), transferred using the PyroMark Vacuum Prep Workstation (QIAGEN) into 40 µL of PyroMark Annealing Buffer containing pyrosequencing primers and then subjected to pyrosequencing reactions using PyroGold Reagents (QIAGEN) following the manufacturer's protocol. The relative proportion of a Q136 variant in a sample was determined using the Allele Quantitation application of the PyroMark ID software.

Serial passage experiments in Madin-Darby canine kidney epithelial cells and eggs
Three isolates containing WT (i.e. Q136) and Q136K or alternatively WT (Q136) and Q136R were passaged four times in either MDCK cells from Queensland Health Scientific Services (henceforth referred to as Brisbane MDCK cells), MDCK cells from the WHO CC, MDCK-SIAT1 cells or 11 day-old embryonated hens' eggs. The Brisbane MDCK cells were originally received from ATCC (CCL-34) and used at passage level 77 to 90 and, before use in the serial viral passage experiments, were grown

Table 1

Frequency of Q136K or Q136R variants detected in A(H1N1)pdm09 or A(H3N2) surveillance samples in the Asia-Pacific region, 2009–2014

Year	A(H1N1)pdm09			A(H3N2)		
	Isolates N	Q136K n (%)	Q136R n (%)	Isolates N	Q136K n (%)	Q136R n (%)
2009	1,068	0 (0.0)	0 (0.0)	117	0 (0.0)	0 (0.0)
2010	1,796	0 (0.0)	0 (0.0)	280	0 (0.0)	0 (0.0)
2011	1,172	9 (0.8)	12 (1.0)	798	1 (0.1)	0 (0.0)
2012	237	1 (0.4)	1 (0.4)	1,620	0 (0.0)	0 (0.0)
2013	960	0 (0.0)	0 (0.0)	668	1 (0.1)	0 (0.0)
2014	1,236	0 (0.0)	0 (0.0)	526	0 (0.0)	0 (0.0)
Total	6,469	10 (0.2%)	13 (0.2)	4,009	2 (<0.1)	0 (0.0)

The Global Initiative on Sharing Avian Influenza Data (GISAID) isolate identity numbers for sequences of selected Q136K and Q136R viruses detected in this study are: EPI_ISL_103643, 172961, 172962, 106853, 106854, 106855, 106856, 106849, 122566, 106851 and 128664 (www.gisaid.org).

in Opti-MEM with L-glutamine (Thermo Fisher), supplemented with 100x Antibiotic / Antimycotic (Thermo Fisher) and 10% fetal bovine serum (Thermo Fisher). The MDCK-SIAT1 cells were kindly provided by Professor Hans-Dieter Klenk, University of Marburg, Germany, and before the serial viral passage study, these cells were passaged in the same medium as described previously for the WHO CC MDCK cells except that it was further supplemented with 1 mg/mL G418 sulphate (Geneticin, GIBCO, US). Viruses were diluted 1:100 after each passage and used for inoculation of the subsequent passage. Viruses were cultured in eggs for two days at 35°C, and in cells for up to five days at 35°C in the medium used for the WHO CC MDCK cells described previously.

Plasmid construction, site directed mutagenesis and reverse genetics
The NA segments from the A(H3N2) virus A/Wyoming/3/2003 and the A(H1N1)pdm09 virus A/Auckland/0.5009 were amplified by RT-PCR, digested with the restriction enzyme *BsaI* and ligated into the *Bsm*BI digested pHW2000 vector (kindly provided by St Jude Children's Research Hospital, Memphis) using the Quick Ligation Kit T4 ligase (New England Biolabs) according to the manufacturer's protocol. Plasmids were transformed into One Shot TOP10 Competent *E. coli* Cells (Life Technologies) and positive clones were inoculated into 5 mL Fast-Media Ampicillin TB (InvivoGen, US) and incubated at 37°C with shaking at 225 rpm for 16 hours. Plasmid DNA was then isolated using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's protocol and eluted into 50 µL autoclaved water.

Single nucleotide (nt) mutations were introduced into the NA plasmids to alter the Q136 codon to residues H, K, L, or R using the QuikChange Multi Site Directed

Neuraminidase (NA) activity and NA inhibitor susceptibility of reverse genetics derived Q136 variants

Subtype	NA mutation	NA activity (% of WT)	IC$_{50}$ (nM) ± SD (Fold difference of IC$_{50}$ compared with WT)			
			Zanamivir	Oseltamivir	Peramivir	Laninamivir
A(H1N1)pdm09	None (WT)	–	0.2±0.0 (–)	0.3±0.1 (–)	0.1±0.0 (–)	0.2±0.0 (–)
	Q136L	54.2±3.6	**6.3±2.0 (32)**	**3.6±0.6 (12)**	0.5±0.3 (5)	0.8±0.1 (4)
	Q136H	98.6±0.8	0.2±0.0 (1)	0.2±0.1 (1)	0.1±0.0 (1)	0.2±0.0 (1)
	Q136R	34.2±0.8	**161.9±35.0 (810)**	0.2±0.0 (1)	**68.2±16.8 (682)**	**131.8±30.7 (659)**
	Q136K	32.9±1.6	**117.7±11.7 (589)**	0.3±0.0 (1)	**34.3±3.7 (343)**	**25.5±6.0 (126)**
A(H3N2)	None (WT)	–	1.3±0.3 (–)	0.5±0.1 (–)	0.3±0.0 (–)	1.2±0.1 (–)
	Q136L	37.3±1.3	**12.8±2.5 (10)**	**6.5±1.2 (13)**	0.9±0.3 (3)	1.3±0.4 (1)
	Q136H	109.3±1.0	0.8±0.2 (1)	0.2±0.1 (1)	0.2±0.1 (1)	1.0±0.2 (1)
	Q136R	82.5±3.6	3.0±0.7 (2)	1.4±0.2 (3)	0.8±0.0 (3)	1.1±0.0 (1)
	Q136K	43.2±3.1	**12.3±2.4 (10)**	**6.6±1.4 (13)**	0.9±0.3 (3)	1.6±0.2 (1)

IC$_{50}$: drug concentration required to inhibit 50% of the NA activity; SD: standard deviation; WT: wildtype.

Figures in bold indicate a 10-fold or greater increase in IC$_{50}$ compared with the respective WT virus. Mean±SD are derived from three separate experiments.

Mutagenesis Kit (Agilent Technologies) and using mutagenesis primers designed according to manufacturer's guidelines and synthesised by GeneWorks (Adelaide, Australia). The NA segment was sequenced directly from the plasmid as described previously to confirm that the desired mutation had been introduced and that no additional mutations were present.

Recombinant viruses composed of the NA gene from one of the viruses described above, and the remaining seven segments from A/Puerto Rico/8/34 (A/PR/8/34 plasmids kindly provided by Dr Robert Webster, St. Jude Children's Research Hospital, Memphis) were generated by reverse genetics. All eight plasmids were transfected into a co-culture of 293T and MDCK cells as previously described [21]. Rescued viruses were subsequently cultured in MDCK cells in maintenance media described above.

Thermostability and neuraminidase cleavage from A549 cells

The thermostability of the NA was determined by subjecting viruses to a range of temperatures and then determining the amount of residual NA activity as a percentage of the NA activity of the virus at 37°C. The lower temperatures (38 and 41°C) were physiologically relevant, while the higher temperatures (47 and 54°C) provided information about protein stability. Virus was incubated at 37, 38, 41, 47 and 54°C for 15 min using the gradient function on a thermocycler (Bio-Rad Laboratories) and the NA activity of each virus calculated based on the mean of triplicate assays.

NA cleavage efficiency was characterised by the binding and elution of WT and mutant virus to A549 adenocarcinomic human alveolar epithelial cells (ATCC) following a previously published protocol [22]. A549 cells were grown in the growth medium described previously. Virus with a known HA titre was allowed to bind

to human epithelial cells at 4°C for 30 min and the HA titre of the supernatant determined. To assess the NA efficiency, the virus/cells (previously incubated at 4°C) were then incubated at 37°C to provide an opportunity for NA to cleave the HA and release the virus from cells. The comparative HA titre of the cell supernatant, without cells (control) compared with 'after binding' (4°C) and 'after elution' (37°C) incubations, was used to indicate the efficiency of NA cleavage.

Results

Q136K and Q136R variants detected during surveillance testing

A total of 6,469 A(H1N1)pdm09 and 4,009 A(H3N2) virus isolates from the Asia-Pacific region were tested at the WHOCC for their susceptibility to the NAIs from 2009 to 2014. Prior to 2011, all A(H1N1)pdm09 and A(H3N2) virus isolates demonstrated normal inhibition to zanamivir. However, in 2011, 21 of 1,172 (1.1%) A(H1N1)pdm09 isolates tested showed either reduced- or highly reduced-inhibition to zanamivir due to a substitution of the Q136 residue in the NA to either K (n=9) or R (n=12) (Table 1). In 2012, two of 237 (0.8%) A(H1N1)pdm09 virus isolates were also considerably less inhibited by zanamivir and similarly contained either a Q136K or Q136R NA substitution. After 2012, no more A(H1N1)pdm09 virus isolates with Q136K or Q136R were detected. With regard to A(H3N2), during the period from 2011 to 2014, zanamivir inhibition was found to be reduced in two virus isolates (one in 2011 and one in 2013) due to a Q136K NA substitution (Table 1).

Only one of the Q136K A(H1N1)pdm09 isolates and three Q136R isolates appeared to be pure viral populations following pyrosequencing analysis, while all of the others were detected as mixed viral populations, with proportions of variant virus ranging from 47 to

80% for the Q136K isolates, and three to 94% in the Q136R isolates. As a result of the mixed viral populations, the zanamivir IC_{50} values of the different isolates differed markedly from each other, with the Q136K isolates ranging from 1.9 to 250 nM, and the Q136R isolates ranging from 2.1 to 92 nM. Neither the Q136K nor Q136R isolates had an increased oseltamivir IC_{50}, although they did have an increased peramivir and laninamivir IC_{50} (data not shown).

Following detection of the Q136K or Q136R variants in MDCK isolates, it was necessary to determine whether viruses with these substitutions were present in the respective original clinical specimens. Of the 25 virus isolates where a Q136K or Q136R variant was detected, 16 clinical specimens were available for analysis. Sanger sequencing or pyrosequencing failed to detect the presence of the variants in any of the clinical specimens tested, even as a minor proportion of the viral population. Clonal analysis of one of the virus samples was conducted but again failed to detect the presence of the variant in 92 clones analysed. These data demonstrated that the Q136K or Q136R variants viruses were either below the level of detection of the assays or were not present in the specimen but spontaneously generated during culture. Given these findings, it was clear that these viruses were being positively selected during MDCK cell culture. All of the Q136K or Q136R isolates had been passaged between two and four times in MDCK cells before analysis. Two Q136R A(H1N1)pdm09 isolates which contained ›80% Q136R had only been passaged twice in MDCK cells. Seventeen (68%) of the 25 Q136K or Q136R isolates detected at the WHO CC, had initially been passaged and submitted to the WHO CC by a single laboratory in Brisbane, Australia. This was a disproportionately high number of viruses with a mutation at Q136 given that during the 2009 to 2014 period this laboratory submitted 24.3% (2,552/10,478) of all of the viruses tested at the WHOCC.

Serial passage of Q136K and Q136R A(H1N1) pdm09 viruses in cell and egg culture

To better understand whether the MDCK cells used by the Brisbane laboratory were more selective for the Q136K or Q136R A(H1N1)pdm09 variants than other cell lines, two isolates containing NA 136 Q/K mixtures (named A/Brisbane/345/2011 and A/Brisbane/70/2011) and one isolate with a NA 136 Q/R mixture (A/Perth/130/2011) were passaged four times in either Brisbane MDCK cells, WHOCC MDCK cells, MDCK-SIAT1 cells or hens eggs. Passage in the Brisbane MDCK cells either maintained or increased the proportion of Q136K or Q136R viruses in the viral population (Figure 1). This positive selection for the Q136K variant was also observed following passage of the A/Brisbane/345/2011 isolate in the WHOCC MDCK cells, but was not seen with the other two isolates, where passage in WHOCC MDCK cells resulted in the gradual loss of the Q136K or Q136R variant. The MDCK-SIAT1 cells consistently selected against the Q136K or Q136R variants, with the proportion of each variant gradually

decreasing after serial passage (Figure 1). The largest change in mixture proportion was seen following egg passage, which showed that growth of the variants were not well supported in embryonated eggs and were rapidly selected against, such that after a single passage in eggs the Q136K and Q136R viruses were undetectable in two of the isolates, and accounted for ‹10% of the viral population in the third isolate (Figure 1).

Neuraminidase activity and neuraminidase inhibitor susceptibility of reverse genetics derived Q136K, R, L and H variants

Examination (in 2014) of human and avian N1 and N2 sequences from the public sequence databases Global Initiative on Sharing Avian Influenza Data (GISAID) and GenBank revealed not only the Q136R and Q136K substitutions, but also Q136L and Q136H, present in a small number (less than 1%) of sequences from A(H3N2), A(H1N1) and A(H5N1) virus isolates. Site directed mutagenesis and reverse genetics were used to better investigate the phenotypic effect of the Q136K, Q136R, Q136L and Q136H substitutions in the NAs from A(H1N1)pdm09 and A(H3N2) viruses. 7:1 reassortants containing either the N1 or N2 WT NA (no mutations) or variant NA (Q136K, R, L or H) on a PR/8 backbone were successfully generated by reverse genetics.

For N1 reassortants, the Q136H mutant retained full NA activity, while the Q136R, Q136K and Q136L mutants had between 33 and 54% of the WT NA activity (Table 2). For the same mutations in the N2 NA, the Q136H substitution also had no effect on NA activity, whereas the Q136R substitution caused a minor reduction in NA activity (82% activity of WT), and the Q136K and Q136L substitutions caused large reductions in NA activity (40% activity of WT) (Table 2).

Analysis of the N1 reassortants for NAI susceptibility showed that the Q136H substitution had no effect, whereas the Q136L mutant demonstrated a moderate 32-fold increase in zanamivir IC_{50} and a minor 4- to 12-fold increase in oseltamivir, peramivir and laninamivir IC_{50} compared with the respective WT. In comparison, the Q136R substitution caused a 659- to 810-fold increase in zanamivir, peramivir and laninamivir IC_{50} compared with WT, while the Q136K substitution caused a 126- to 589-fold increase against the same NAIs (Table 2). Both the Q136K and Q136R substitutions had no effect on oseltamivir susceptibility.

The large effect of the Q136R and Q136K substitutions observed in the N1 NA was not observed in the N2 NA. Q136R caused only a minor (two- to threefold) change in oseltamivir, peramivir and zanamivir IC_{50}. A Q136L or a Q136K substitution caused a moderate 10- to 13-fold increase in zanamivir and oseltamivir IC_{50} and a threefold increase in peramivir IC_{50} but no change in laninamivir sensitivity compared with the WT. Q136H in N2 had no effect on NAI sensitivity, similar to that observed in the N1 NAs (Table 2).

Viral function of reverse genetics derived Q136K, R, L and H variants

The thermostability and HA/NA balance of the reassortant variant viruses were determined. The Q136L and Q136H N1 variants retained high NA activity across the 38°C to 54°C temperature range, equivalent to that of the WT virus. However the N1 reassortants with Q136K and Q136R mutations showed a substantial loss of activity at 54°C (67% and 22% remaining activity compared to 37 °C respectively) (Figure 2A). The N2 reassortant WT had some loss of activity at 54°C (30% remaining activity), as did the NAs with Q136R and Q136H mutations (8–21% remaining activity), while the NAs with Q136K and Q136L mutations maintained high activity (>80%) across the entire temperature range (Figure 2B).

All N1 and N2 reassortant viruses contained the PR/8 HA and all showed good cell binding at 4°C, as indicated by low viral titres in the supernatant. After incubation at 37°C, the N1 WT reassortant and the Q136L variant showed full restoration of HA titre, demonstrating an active NA enzyme, whereas the Q136K and Q136R variants had only partial restoration of HA titre, suggesting that the NA activity was insufficient to cleave all bound virus from the cells (Figure 3A). All of the N2 reassortants, with the exception of Q136K, showed full restoration of HA titre following incubation at 37°C (Figure 3B).

Discussion

In this study we describe the detection of A(H1N1) pdm09 influenza virus isolates, and to a lesser extent A(H3N2) viruses, with amino acid substitutions at the Q136 NA residue that reduce zanamivir, peramivir and laninamivir susceptibility. Surveillance data show that the Q136K and Q136R substitutions occurred sporadically in A(H1N1)pdm09 cultured isolates, with periods such as 2011 where a relatively high detection rate was observed, compared with other years where they were absent. Importantly, in all cases, the residue substitution that was present in the isolate, was not detected in the virus from the clinical specimen demonstrating that the mutation was either arising, or being selected for, during MDCK cell culture passage. Because the 'gold-standard' for laboratory assessment of NAI susceptibility is the phenotypic NA inhibition assay, which requires a cell culture isolate for testing, there is concern that viruses such as these can be reported as being 'resistant' when in fact the virus that came from the patient was sensitive. In addition, the process of cell culture may also select against a resistant virus, meaning that a variant virus is not detected when it was present in the clinical specimen. Misdiagnosis can have an impact on the therapies being used in patient management and may unnecessarily result in therapy being stopped, modified or inappropriately continued. In addition to the Q136K/R variants described here, there are many other NA mutations that alter NAI susceptibility and also appear to be selected during MDCK cell culture [19,23]. Interestingly these seem to be increasingly reported for influenza B viruses [23-25].

Therefore sequence analysis of the influenza viruses in the original specimen remains important when laboratories detect mutations in cultured isolates.

While conventional MDCK cells selected for the Q136K and Q136R A(H1N1)pdm09 variants, their growth was not supported in eggs, with a single passage resulting in the near complete loss of the variant virus. MDCK-SIAT1 cells also did not appear to give selective growth advantage to the Q136 variants. MDCK-SIAT1 cells have enhanced binding due to an increased concentration of α2,6-linked sialic acids on the MDCK cell surface [26], which may mean that viruses with reduced NA activity, such as the Q136K/R variants, have reduced replication in this cell line, possibly explaining the difference with the conventional MDCK cell lines. If clinical samples are available in the future that have been shown to result in MDCK isolates with Q136K/R mutations, it would be useful to test whether primary isolation into MDCK-SIAT1 or human bronchial epithelial cells prevents this initial selection of the variant virus.

Although the Q136K and Q136R variants detected here were all cell culture derived variants, other studies have reported mutations at Q136 that were detected in viruses from clinical specimens. The Q136K mutation was detected in an A(H3N2) virus, together with an E119V NA mutation, in a patient who had previously undergone a bone marrow transplant, following a treatment course of both inhaled and intravenous zanamivir and oseltamivir [27]. Mutations at Q136 have also been detected in ferrets infected with influenza A(H5N1). The Q136L variant was detected in the nasal wash of a zanamivir treated ferret infected with an A(H5N1) virus [28], while a Q136H mutation was detected in an A(H5N1) virus from a ferret not being treated with an antiviral [29]. These reports demonstrate that viruses with these mutations have the potential to infect or replicate in vivo both in the presence or absence of zanamivir pressure.

The zanamivir concentration in sputum 12 hours post-inhalation has been reported to be between 159 and 4,315 nM [30]. Therefore, while the correlation between the drug concentration in sputum and the drug concentration at the site of viral replication is not clear, it is anticipated that only the Q136K and Q136R mutations in the A(H1N1)pdm09 virus may potentially impact the clinical effectiveness of zanamivir. The Q136L mutation in both N1 and N2 NAs and Q136K in N2 NA caused mild increases in zanamivir and oseltamivir IC_{50}, which are expected to be below the concentrations present at the sites of replication in treated individuals [30,31].

An evaluation of the ability of the Q136K and Q136R A(H1N1)pdm09 variants to replicate and transmit in animal models will provide useful insights into the potential risk that these viruses may pose to public health. Where possible these future studies would benefit from using Q136K and Q136R variants that were naturally occurring, rather than cell culture derived or

generated by reverse genetics. One limitation of this study is that the N1 and N2 NAs with Q136 substitutions were assessed in 1:7 reassortant viruses generated by reverse genetics on a PR/8 backbone, therefore there is potential that the HA/NA balance between the variant NA molecules and the HA from PR/8 may be different from that seen in the 'natural' isolate. The in vitro assays showed that as a result of the Q136K mutation the N1 reassortant had a moderate loss in NA activity and thermostability. A reduction in NA enzyme activity and surface expression due to the Q136K NA mutation has been previously reported [32,33]. Pizzorno et al. [33] also demonstrated that an A(H1N1)pdm09 Q136K variant had compromised replication compared with a WT virus in mice, while in a ferret model the variant was able to transmit between contact ferrets, but at slower rate than for the WT virus. However, a study in guinea pigs found that the Q136K variant did not transmit between animals [32]. Taken together, these studies indicate that the replication and transmissibility of the Q136K variant in the A(H1N1)pdm09 virus appears to be compromised and therefore is unlikely to circulate through the human population. However, compensatory mutations in the NA or other genes that may occur in the A(H1N1)pdm09 virus in the future may buffer the compromising effect of the Q136K mutation and improve overall replication and transmissibility of the variant, in a manner similar to that seen for the H275Y mutation [7,34]. To date there has been no evaluation of the in vivo fitness of the Q136R A(H1N1) pdm09 variant.

In this study we have highlighted the challenges that cell culture derived mutations, such as Q136K and Q136R, can pose to the analysis and interpretation of viruses for NAI susceptibility. This further reaffirms the need to sequence viruses from the clinical specimens of any isolate that shows reduced susceptibility in a phenotypic NA inhibition assay to avoid misdiagnosis and any unnecessary change in patient management with respect to the use of antivirals. Our findings highlight the effect of mutations at the Q136 residue of N1 viruses on laninamivir, peramivir or zanamivir susceptibility, and therefore close monitoring of viruses for these mutations in patients being treated with these antivirals is important.

Conflict of interest

None declared.

Authors' contributions

SKL and ACH generated antiviral susceptibility data for circulating viruses submitted to the WHO CC; KL, JB, CB, BH and ACH conducted and analysed the mutagenesis, reverse genetics and functional assays of the variant viruses; KL and ACH wrote the draft of the manuscript and all authors further edited the manuscript and approved the final version of the paper. JM, IGB and ACH provided supervisory oversight.

References

1. Gubareva LV, Kaiser L, Hayden FG. Influenza virus neuraminidase inhibitors.Lancet. 2000;355(9206):827-35. DOI: 10.1016/S0140-6736(99)11433-8 PMID: 10711940

2. Hurt AC, Ho HT, Barr I. Resistance to anti-influenza drugs: adamantanes and neuraminidase inhibitors.Expert Rev Anti Infect Ther. 2006;4(5):795-805. DOI: 10.1586/14787210.4.5.795 PMID: 17140356

3. Hurt AC, Holien JK, Parker MW, Barr IG. Oseltamivir resistance and the H274Y neuraminidase mutation in seasonal, pandemic and highly pathogenic influenza viruses.Drugs. 2009;69(18):2523-31. DOI: 10.2165/11531450-000000000-00000 PMID: 19943705

4. Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic sites in influenza virus neuraminidase.Nature. 1983;303(5912):41-4. DOI: 10.1038/303041a0 PMID: 6188957

5. Yen H-L, Herlocher LM, Hoffmann E, Matrosovich MN, Monto AS, Webster RG, et al. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. Antimicrob Agents Chemother. 2005;49(10):4075-84. DOI: 10.1128/AAC.49.10.4075-4084.2005 PMID: 16189083

6. Hurt AC, Nor'e SS, McCaw JM, Fryer HR, Mosse J, McLean AR, et al. Assessing the viral fitness of oseltamivir-resistant influenza viruses in ferrets, using a competitive-mixtures model. J Virol. 2010;84(18):9427-38. DOI: 10.1128/JVI.00373-10 PMID: 20631138

7. Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance.Science. 2010;328(5983):1272-5. DOI: 10.1126/science.1187816 PMID: 20522774

8. Neuraminidase Inhibitor Flu Treatment Investigator Group,Nicholson KG, Aoki FY, Osterhaus AD, Trottier S, Carewicz O, Mercier CH, et al. . Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial.Lancet. 2000;355(9218):1845-50. DOI: 10.1016/S0140-6736(00)02288-1 PMID: 10866439

9. US Oral Neuraminidase Study Group,Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis R, Riff D, et al. . Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial.JAMA. 2000;283(8):1016-24. DOI: 10.1001/jama.283.8.1016 PMID: 10697061

10. Whitley RJ, Hayden FG, Reisinger KS, Young N, Dutkowski R, Ipe D, et al. Oral oseltamivir treatment of influenza in children. Pediatr Infect Dis J. 2001;20(2):127-33. DOI: 10.1097/00006454-200102000-00002 PMID: 11224828

11. Tamura D, Sugaya N, Ozawa M, Takano R, Ichikawa M, Yamazaki M, et al. Frequency of drug-resistant viruses and virus shedding in pediatric influenza patients treated with neuraminidase inhibitors. Clin Infect Dis. 2011;52(4):432-7. DOI: 10.1093/cid/ciq183 PMID: 21248368

12. Hurt AC, Ernest J, Deng YM, Iannello P, Besselaar TG, Birch C, et al. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. Antiviral Res. 2009;83(1):90-3. DOI: 10.1016/j.antiviral.2009.03.003 PMID: 19501261

13. European Influenza Surveillance Scheme,Meijer A, Lackenby A, Hungnes O, Lina B, van-der-Werf S, Schweiger B, et al. . Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season.Emerg Infect Dis. 2009;15(4):552-60. DOI: 10.3201/eid1504.181280 PMID: 19331731

14. Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus.J Infect Dis. 1998;178(5):1257-62. DOI: 10.1086/314440 PMID: 9780244

15. Wetherall NT, Trivedi T, Zeller J, Hodges-Savola C, McKimm-Breschkin JL, Zambon M, et al. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J Clin Microbiol. 2003;41(2):742-50. DOI: 10.1128/JCM.41.2.742-750.2003 PMID: 12574276

16. van der Vries E, Stelma FF, Boucher CA. Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus.N Engl J

Med. 2010;363(14):1381-2. DOI: 10.1056/NEJMc1003749 PMID: 20879894

17. Nguyen HT, Fry AM, Loveless PA, Klimov AI, Gubareva LV. Recovery of a multidrug-resistant strain of pandemic influenza A 2009 (H1N1) virus carrying a dual H275Y/I223R mutation from a child after prolonged treatment with oseltamivir.Clin Infect Dis. 2010;51(8):983-4. DOI: 10.1086/656439 PMID: 20858074

18. Eshaghi A, Patel SN, Sarabia A, Higgins RR, Savchenko A, Stojios PJ, et al. Multidrug-resistant pandemic (H1N1) 2009 infection in immunocompetent child. Emerg Infect Dis. 2011;17(8):1472-4.PMID: 21801626

19. Okomo-Adhiambo M, Nguyen HT, Sleeman K, Sheu TG, Deyde VM, Garten RJ, et al. Host cell selection of influenza neuraminidase variants: implications for drug resistance monitoring in A(H1N1) viruses. Antiviral Res. 2010;85(2):381-8. DOI: 10.1016/j.antiviral.2009.11.005 PMID: 19917319

20. Hurt AC, Holien JK, Parker M, Kelso A, Barr IG. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation.J Virol. 2009;83(20):10366-73. DOI: 10.1128/JVI.01200-09 PMID: 19641000

21. Hoffmann E, Krauss S, Perez D, Webby R, Webster RG. Eight-plasmid system for rapid generation of influenza virus vaccines.Vaccine. 2002;20(25-26):3165-70. DOI: 10.1016/S0264-410X(02)00268-2 PMID: 12163268

22. Reading PC, Pickett DL, Tate MD, Whitney PG, Job ER, Brooks AG. Loss of a single N-linked glycan from the hemagglutinin of influenza virus is associated with resistance to collectins and increased virulence in mice.Respir Res. 2009;10(117):117. DOI: 10.1186/1465-9921-10-117 PMID: 19930664

23. Leang S-K, Deng YM, Shaw R, Caldwell N, Iannello P, Komadina N, et al. Influenza antiviral resistance in the Asia-Pacific region during 2011. Antiviral Res. 2013;97(2):206-10. DOI: 10.1016/j.antiviral.2012.12.016 PMID: 23274624

24. Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, Bright RA, et al. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob Agents Chemother. 2008;52(9):3284-92. DOI: 10.1128/AAC.00555-08 PMID: 18625765

25. Escuret V, Collins PJ, Casalegno JS, Vachieri SG, Cattle N, Ferraris O, et al. A novel I221L substitution in neuraminidase confers high-level resistance to oseltamivir in influenza B viruses. J Infect Dis. 2014;210(8):1260-9. DOI: 10.1093/infdis/jiu244 PMID: 24795482

26. Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk HD. Overexpression of the α-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors.J Virol. 2003;77(15):8418-25. DOI: 10.1128/JVI.77.15.8418-8425.2003 PMID: 12857911

27. Eshaghi A, Shalhoub S, Rosenfeld P, Li A, Higgins RR, Stogios PJ, et al. Multiple influenza A (H3N2) mutations conferring resistance to neuraminidase inhibitors in a bone marrow transplant recipient. Antimicrob Agents Chemother. 2014;58(12):7188-97. DOI: 10.1128/AAC.03667-14 PMID: 25246391

28. Hurt AC, Lowther S, Middleton D, Barr IG. Assessing the development of oseltamivir and zanamivir resistance in A(H5N1) influenza viruses using a ferret model.Antiviral Res. 2010;87(3):361-6. DOI: 10.1016/j.antiviral.2010.06.009 PMID: 20603155

29. Govorkova EA, Ilyushina NA, Marathe BM, McClaren JL, Webster RG. Competitive fitness of oseltamivir-sensitive and -resistant highly pathogenic H5N1 influenza viruses in a ferret model.J Virol. 2010;84(16):8042-50. DOI: 10.1128/JVI.00689-10 PMID: 20519385

30. Peng AW, Milleri S, Stein DS. Direct measurement of the anti-influenza agent zanamivir in the respiratory tract following inhalation.Antimicrob Agents Chemother. 2000;44(7):1974-6. DOI: 10.1128/AAC.44.7.1974-1976.2000 PMID: 10858364

31. Calfee DP, Peng AW, Cass LM, Lobo M, Hayden FG. Safety and efficacy of intravenous zanamivir in preventing experimental human influenza A virus infection.Antimicrob Agents Chemother. 1999;43(7):1616-20.PMID: 10390212

32. Kaminski MM, Ohnemus A, Staeheli P, Rubbenstroth D. Pandemic 2009 H1N1 influenza A virus carrying a Q136K mutation in the neuraminidase gene is resistant to zanamivir but exhibits reduced fitness in the guinea pig transmission model.J Virol. 2013;87(3):1912-5. DOI: 10.1128/JVI.02507-12 PMID: 23192869

33. Pizzorno A, Abed Y, Rhéaume C, Bouhy X, Boivin G. Evaluation of recombinant 2009 pandemic influenza A (H1N1) viruses harboring zanamivir resistance mutations in mice and ferrets. Antimicrob Agents Chemother. 2013;57(4):1784-9. DOI: 10.1128/AAC.02269-12 PMID: 23357777

34. Butler J, Hooper KA, Petrie S, Lee R, Maurer-Stroh S, Reh L, et al. Estimating the fitness advantage conferred by permissive neuraminidase mutations in recent oseltamivir-resistant A(H1N1)pdm09 influenza viruses. PLoS Pathog. 2014;10(4):e1004065. DOI: 10.1371/journal.ppat.1004065 PMID: 24699865