# The role of balanced haemagglutinin-neuraminidase activity in the genesis of transmissible neuraminidase inhibitor-resistant variants in seasonal and novel pandemic influenza A H1N1 viruses

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KEY MESSAGES

1. The H275Y mutation reduced neuraminidase enzyme activity, increased neuraminidase $K_M$ for 3'-sialyllactose or 6'-sialyllactose, decreased viral infectivity in mucin-secreting human airway epithelial cells, and attenuated pathogenicity in ferrets, when compared with its wild-type counterparts.
2. All H275Y variants of recombinant A(H1N1) pdm09 or seasonal H1N1 influenza viruses with different haemagglutinin-neuraminidase gene constellations were transmitted from inoculated ferrets to naïve direct contact or respiratory droplet contact ferrets, with the transmission efficiency minimally affected, when compared with their wild-type counterparts.

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## Introduction

Since 2009, the neuraminidase (NA) inhibitors have become the major weapon against seasonal H3N2 and the A(H1N1)pdm09 influenza viruses, as these viruses are largely resistant to the M2 ion channel blockers. Mutations that confer resistance to NA inhibitors at the NA catalytic or framework sites may impair enzyme function and compromise viral fitness. The fitness of the NA inhibitor-resistant variants can also be confounded by the haemagglutinin (HA) protein that possesses a counteracting activity to the NA protein. Resistance to NA inhibitors among circulating seasonal influenza viruses was low until 2007 when a H275Y variant emerged among seasonal H1N1 viruses and spread globally by the end of 2008. The A/Brisbane/59/07 (Brisbane)-like virus associated with the H275Y NA mutation had undergone a major antigenic drift from the previously circulating A/New Caledonia/20/99 (NewCal)-like virus.[1] The emergence of the epistatic mutations V234M and R222Q may increase surface NA expression and viral fitness while accommodating the H275Y NA mutation that emerged.[2]

The A(H1N1)pdm09 virus may acquire resistance to NA inhibitors through spontaneous NA mutation or via genetic reassortment with the seasonal H1N1 virus. To achieve a comprehensive understanding of the various degrees of NA functional loss that has been implicated by the H275Y NA mutation, we systematically generated three pairs of recombinant A(H1N1)pdm09 viruses with their NA genes derived from the CA04, NewCal, or Brisbane viruses. Viral fitness was evaluated using different in vitro and in vivo models. We also evaluated the effect of HA and NA derived from the Brisbane-like viruses, including the permissive V234M and R222Q NA mutations. This study provided an in vitro and in vivo comparison of the effect of the H275Y mutation across three antigenic strains exhibiting different epidemiological outcome.[3]

This study aimed to (1) evaluate the fitness and transmission potential of NA inhibitor-resistant pandemic H1N1 2009 virus with spontaneous NA mutations that confer resistance to NA inhibitors or with NA gene derived from the Brisbane-like oseltamivir-resistant viruses; (2) determine the effect of HA and NA of Brisbane-like oseltamivir-resistant seasonal H1N1 viruses on transmissibility; and (3) investigate the potential effect of the secondary permissive mutations (R222Q and V234M).

## Methods

This study was conducted from January 2010 to June 2012.

### Generation of recombinant viruses

The genome of A(H1N1)pdm09 or seasonal H1N1 influenza viruses were amplified by RT-PCR and cloned into pHW2000 plasmid. The H275Y NA mutation was introduced to the plasmids using site-directed mutagenesis.

### Competitive growth between A(H1N1) pdm09 virus with or without the NA-H275Y mutation in cells

RG-CA04 and RG-CA04$^{NA\text{-}H275Y}$ were mixed in different ratios to infect pre-washed differentiated normal human bronchial epithelial (NHBE) cells or MDCK-SIAT1 cells (with or without 0.2 μM oseltamivir carboxylate). RNA was extracted at different times post-infection and the NA gene was amplified by RT-PCR and cloned into pCR4-TOPO vector to determine the ratio between the H275 and Y275 genotypes by Sanger sequencing.

### NA kinetics

NA kinetics using 20-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) and sialosides [3'sialyllactose (3'SL) and 6'sialyllactose (6'SL)] were determined with viruses standardised to 5000 or $1.74\times10^6$ PFU/mL, respectively. The data were fitted using nonlinear regression to determine the Michaelis constant ($K_M$) and maximum velocity ($V_{max}$) of substrate conversion.

### Transmission experiments in ferrets

Transmissibility was evaluated in 4-6 month old male ferrets seronegative for influenza A NP protein by ELISA (ID.vet) and influenza B virus (B/Brisbane/60/08) by HA inhibition assay (≤20). All studies were conducted in compliance with applicable laws and with ethics approval. Donor ferrets were inoculated with $10^4$ PFU of recombinant virus intra-nasally under isoflurane anaesthesia. At 1 day post-infection, naïve direct contact and respiratory droplet contact ferrets were introduced. Nasal washes were collected from all ferrets on alternate days for 14 days to monitor virus shedding.

## Results

### H275Y NA mutation led to differential NA functional loss in different NA backgrounds

Recombinant viruses carrying the H275Y mutation showed lower $V_{max}$ and higher $K_M$ values than their wild-type counterparts (Table). The $K_M$ value of the RG-CA04×Brisbane$^{NA}$ for MUNANA, 3'SL, or 6'SL was significantly lower than that of the RG-CA04×NewCal$^{NA}$ or the RG-CA04 viruses (Table). Overall, the H275Y mutation led to decreased NA activity and increased $K_M$ for NAs derived from seasonal or A(H1N1)pdm09 H1N1 influenza viruses.

### Recombinant viruses carrying the H275Y mutation were compromised in establishing infection in mucin-secreting differentiated NHBE cells

We compared the ability of the recombinant viruses to infect mucin-secreting differentiated NHBE cells in the presence or absence of the mucin layer by washing the cells extensively or leaving them unwashed prior to infection. Among the pre-washed cells, all wild-type viruses (RG-CA04, RG-CA04×NewCal$^{NA}$, and RG-CA04×Brisbane$^{NA}$) were able to establish infection in 4/4 replicates; the H275Y variants, RG-CA04$^{NA\text{-}H275Y}$, RG-CA04×NewCal$^{NA\text{-}H275Y}$, RG-CA04×Brisbane$^{NA\text{-}H275Y}$, established infection in 3/4, 3/4, and 2/4 replicates, respectively. Comparable viral titres were observed between the replicates successfully infected by the wild-type and the H275Y counterparts. In cells that were left unwashed prior to infection, the wild-type viruses could establish infection in 4/5, 5/5, and 3/5 replicates of the differentiated NHBE respectively, whereas their H275Y variants could establish infection in only 2/5, 1/5, and 1/5 replicates respectively. Overall, recombinant viruses with the H275Y mutation, regardless of the origin of the NA gene, exhibited a reduced ability to establish infection in the differentiated NHBE cells.

### Competitive growth between RG-CA04 and RG-CA04$^{H275Y}$ viruses in vitro

RG-CA04 and RG-CA04$^{NA\text{-}H275Y}$ viruses were pre-mixed at different ratios to co-infect the washed differentiated NHBE cells or MDCK-SIAT1 cells. RG-CA04 genotype increased over time but was

**TABLE. Neuraminidase enzyme kinetics using MUNANA, 3'-SL, and 6'-SL substrates** (Permission from: Wong DD, Choy KT, Chan RW, et al. Comparable fitness and transmissibility between oseltamivir-resistant pandemic 2009 and seasonal H1N1 influenza viruses with the H275Y neuraminidase mutation. J Virol 2012;86:10558-70.)

| Virus | MUNANA | | | 3'SL | | | 6'SL | | |
|---|---|---|---|---|---|---|---|---|---|
| | $K_M$ (μM) | $V_{max}$ | $V_{max}$ ratio | $K_M$ (μM) | $V_{max}$ | $V_{max}$ ratio | $K_M$ (μM) | $V_{max}$ | $V_{max}$ ratio |
| RG-CA04 | 26.5 | 4.0 | 1.0 | 622.7 | 101.0 | 1.0 | 3469 | 68.2 | 1.0 |
| RG-CA04$^{NA\text{-}H275Y}$ | 61.9 | 3.5 | 0.9 | 905.3 | 105.8 | 1.1 | 4224 | 57.8 | 0.8 |
| RG-CA04×NewCal$^{NA}$ | 42.3 | 4.3 | 1.0 | 741.5 | 111.3 | 1.0 | 2355 | 84.5 | 1.0 |
| RG-CA04×NewCal$^{NA\text{-}H275Y}$ | 84.5 | 2.4* | 0.6 | 1370.0 | 69.5* | 0.6 | 3987 | 57.2 | 0.7 |
| RG-CA04×Brisbane$^{NA}$ | 14.0† | 6.8 | 1 | 454.2 | 152.0 | 1.0 | 2108 | 105.6 | 1.0 |
| RG-CA04×Brisbane$^{NA\text{-}H275Y}$ | 26.4 | 4.3* | 0.6 | 698.9 | 97.5* | 0.6 | 2462 | 67.8* | 0.6 |

* P<0.05 when compared to counterpart wild-type viruses

† P<0.05 when compared to RG-CA04×NewCal$^{NA}$ virus

not able to completely dominate the oseltamivir-resistant Y275 genotype in the MDCK-SIAT1 cells. Overall, the RG-CA04 exhibited a slightly higher survival advantage than RG-CA04$^{NA\text{-}H275Y}$ virus when co-infected at different ratios in the absence of oseltamivir (Fig 1). In the absence of oseltamivir carboxylate, the proportion of RG-CA04 genotype decreased progressively over time.

### Transmissibility of the recombinant A(H1N1)pdm09 viruses in ferrets

RG-CA04 caused greater levels of weight loss in ferrets compared with the RG-CA04$^{NA\text{-}H275Y}$ or RG-CA04×Brisbane$^{NA\text{-}H275Y}$ viruses (Fig 2). One of the two ferrets inoculated with RG-CA04 virus died on day 8 post-inoculation, whereas all ferrets survived in the RG-CA04$^{NA\text{-}H275Y}$ and RG-CA04×Brisbane$^{NA\text{-}H275Y}$ groups. Transmission from inoculated ferret to naïve direct contact and respiratory droplet contact ferrets was observed for all three recombinant CA04 viruses at day 4 post-inoculation (Fig 2). Overall, we observed that RG-CA04$^{NA\text{-}H275Y}$ or RG-CA04×Brisbane$^{NA\text{-}H275Y}$ viruses possessed attenuated pathogenicity but retained comparable transmission efficiency to the RG-CA04 virus.

### Generation of recombinant seasonal H1N1 viruses with different HA and NA constellations

A series of recombinant viruses with identical internal genes but with different HA-NA gene constellation derived from NewCal and Brisbane were generated. Permissive mutations R222Q and V234M were introduced into the NA of the NewCal virus. All recombinant viruses replicated to comparable titres in MDCK cells. H275Y NA mutation consistently led to an increased $K_M$ values for MUNANA, 3'SL, or 6'SL substrates. Interestingly, we observed that the permissive mutations R222Q and V234M did not significantly affect the $K_M$ value of the wild-type NewCal NA, whereas the $K_M$ values of RG-NewCal$^{HA,\ NA\text{-}R222Q,V234M,\ H275Y}$ were lower than that of the RG-NewCal$^{HA,NA\text{-}H275Y}$ virus.

### Transmissibility of the recombinant seasonal H1N1 viruses in ferrets

The transmissibility of RG-Brisbane$^{HA,NA\text{-}H275Y}$, RG-NewCal$^{HA,NA}$, RG-NewCal$^{HA,NA\text{-}H275Y}$, or RG-NewCal$^{HA}$×Brisbane$^{NA\text{-}H275Y}$ recombinant seasonal H1N1 viruses was evaluated. All viruses could transmit to direct contact and respiratory droplet contact ferrets with minor differences in efficiency. By comparing the RG-NewCal$^{HA,NA}$ and RG-NewCal$^{HA,NA\text{-}H275Y}$ viruses, we observed that the H275Y NA mutation slightly decreased the respiratory droplet transmissibility but not the direct contact transmissibility.

## Discussion

The H275Y mutation led to reduced NA enzyme function, regardless of the origin of the NA gene segment. The NA of the Brisbane virus of which the H275Y variant spread globally exhibited a unique NA enzyme property compared with the NA derived from the NewCal or CA04 viruses. Specifically, the

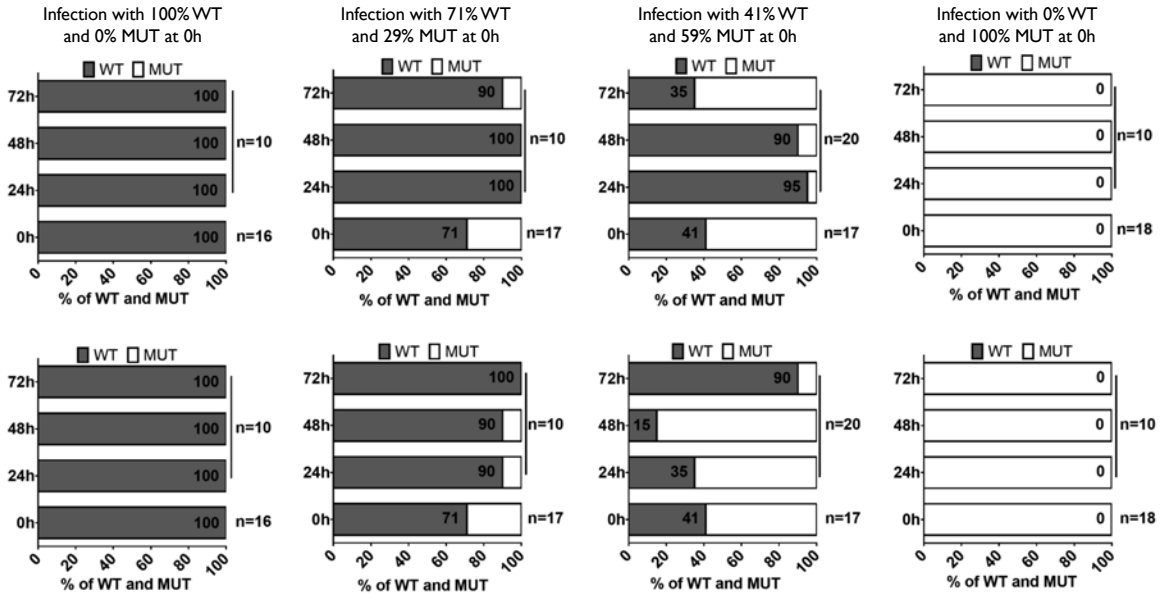


**FIG 1. Competitive replication of RG-CA04 and RG-CA04$^{NA\text{-}H275Y}$ in differentiated NHBE cells** (Permission from: Wong DD, Choy KT, Chan RW, et al. Comparable fitness and transmissibility between oseltamivir-resistant pandemic 2009 and seasonal H1N1 influenza viruses with the H275Y neuraminidase mutation. J Virol 2012;86:10558-70.)

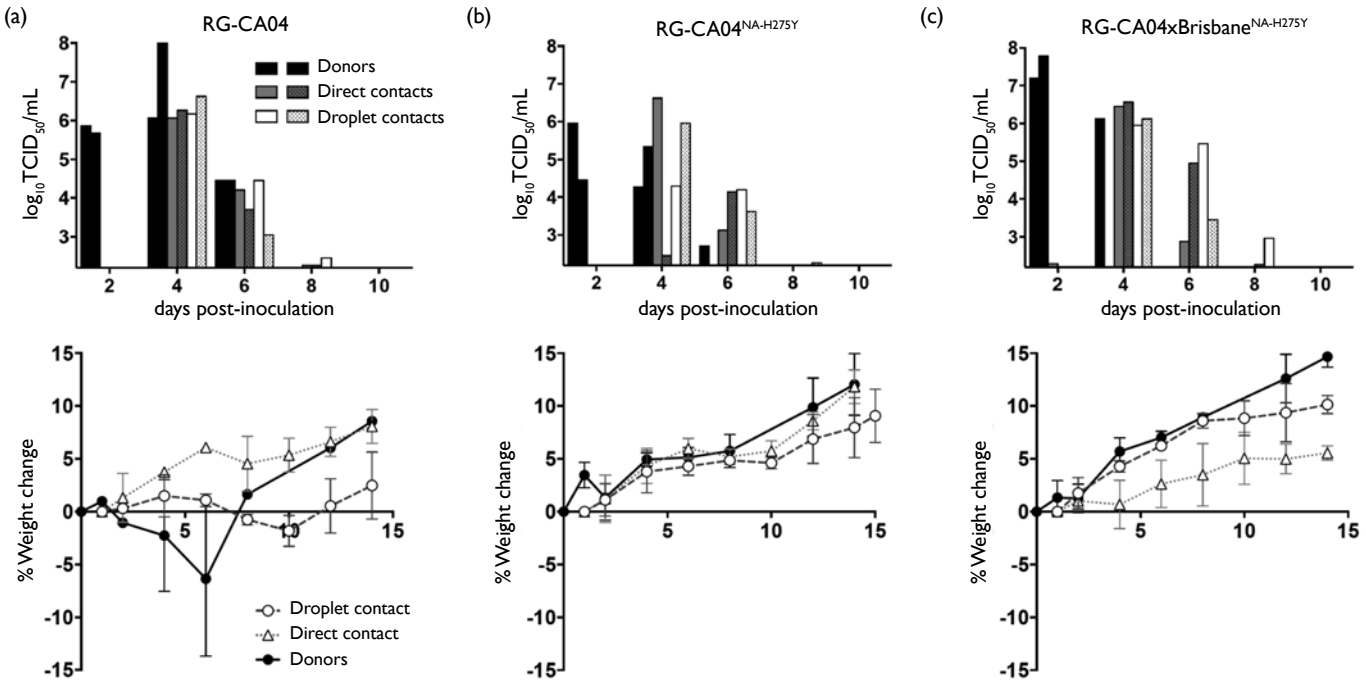


**FIG 2. Transmission of (a) RG-CA04, (b) RG-CA04$^{NA-H275Y}$, and (c) RG-CA04x Brisbane$^{NA-H275Y}$ viruses in ferrets:** viral titres ($log_{10}TCID_{50}$/mL) and percentage of ferret weight changes for donor ferrets, direct contacts, and respiratory droplet contacts. (Permission from: Wong DD, Choy KT, Chan RW, et al. Comparable fitness and transmissibility between oseltamivir-resistant pandemic 2009 and seasonal H1N1 influenza viruses with the H275Y neuraminidase mutation. J Virol 2012;86:10558-70.)

$K_M$ value of oseltamivir-sensitive Brisbane NA was consistently lower in catalysing MUNANA, 3'SL, or 6'SL when compared with the NewCal or CA04 NA, consistent with a previous study.[4] Introduction of the permissive R222Q and V234M mutations[2] into the NewCal NA did not significantly increase NA activity or the $K_M$ value. The H275Y variants with reduced NA function showed decreased infectivity in the mucin-secreting differentiated NHBE cells. Direct competitive assay between the RG-CA04 and RG-CA04$^{NA-H275Y}$ variant suggested that the oseltamivir-sensitive RG-CA04 virus showed minor survival advantage over the H275Y variant in differentiated NHBE or in MDCK-SIAT1 cells.

Despite the detection of reduced viral competencies, both the oseltamivir-sensitive and the oseltamivir-resistant H275Y variants in recombinant A(H1N1)pdm09 viruses or the seasonal H1N1 viruses with different HA-NA constellations were able to transmit to naïve direct contact or respiratory droplet contact ferrets. Our results suggest that the H275Y mutation in H1N1 influenza leads to a minor reduction in viral fitness with its transmission potential being minimally affected in the naïve ferret model.

## Conclusion

The oseltamivir-resistant H275Y variants have transmission potential; continued monitoring of this mutation among circulating A(H1N1)pdm09 strains is important.

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