
Emergence of Oseltamivir- Resistant Pandemic (H1N1) 2009 Virus within 48 Hours

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An oseltamivir-resistant influenza A pandemic (H1N1) 2009 virus evolved and emerged from zero to 52% of detectable virus within 48 hours of a patient's exposure to oseltamivir. Phylogenetic analysis and data gathered by pyrosequencing and cloning directly on clinical samples suggest that the mutant emerged *de novo*.

Early descriptions of emergence of H275Y mutants in pandemic (H1N1) 2009 virus showed resistance after 11 and 23 days of therapy in immunosuppressed patients (1). Also in previous reports, transmission of mutant viruses occurred in immunosuppressed patients (2), although a cluster among healthy persons demonstrated that H275Y mutants could replicate and cause disease in the absence of drug pressure (3). Additional reports noted decreasing times to detection of resistance, from 14 to 4 days after therapy (4–6). We report development of oseltamivir-resistant pandemic (H1N1) 2009 virus in an infected woman in Singapore within 48 hours of drug treatment.

The Study

Pandemic (H1N1) 2009 virus was first detected in Singapore in May 2009. Infected patients were placed in isolation and offered oseltamivir, and respiratory samples were collected for screening for H275Y, the principal mutation associated with oseltamivir resistance in influenza A N1 viruses. H275Y was detected in a pandemic (H1N1)

2009 virus isolated from a sample from a 28-year-old female patient on the sixth day of illness within 48 hours of her exposure to oseltamivir. (Written patient consent was obtained under Review Board approval no. E09-230.) A sore throat, myalgia, redness of the right eye, and a mild fever with a productive cough had developed on the day she returned to Singapore from Hawaii. Eleven close contacts, exposed before emergence of the mutant, were given oseltamivir prophylaxis on the patient's fourth day of treatment, and they remained well. By performing sequencing directly on 6 of her respiratory samples and on their viral isolates (online Technical Appendix, www.cdc.gov/EID/content/16/10/1633-Techapp.pdf), we investigated the origin of this H275Y mutant (the second earliest sample of this mutation to be deposited in GenBank).

Only wild-type sequences were detected in samples collected on the day before, the day of, and 14 hours after initiation of oseltamivir therapy (online Technical Appendix Table 1). Similarly, only wild-type sequences were detected in 192 clones generated from a sample collected a few hours before initiation of oseltamivir. Pyrosequencing directly on clinical samples collected 38 and 45 hours after initiation of therapy showed 24% and 52% mutant sequences, respectively (Figure). The relative amount of virus detected, as determined by the strength of PCR results (online Technical Appendix Table 1), increased from days 3 to 5 of illness by $\approx 1,000$ -fold. Oseltamivir treatment was initiated on day 4 of illness. On the same day, her maximum body temperature (38.8°C) was recorded, although no other signs or symptoms of clinical deterioration were observed. Her fever resolved on day 5 of illness, and she was allowed out of isolation on day 7 of illness.

When we compared the mutant drug-resistant isolate GN285 with the wild-type drug-sensitive isolate ON129, we found only 1 aa difference, the H275Y resistance-causing mutation in the neuraminidase gene, whereas a comparison of GN285 and ON129 with the reference strain A/Texas/05/2009(H1N1) showed several mutations (online Technical Appendix Table 2). Mutation PB1 I435V, shared between GN285 and ON129, did not occur in any of the other 7 drug-resistant strains included in the analysis. The whole genome maximum likelihood tree (online Technical Appendix Figure 1) showed that the wild-type and resistant viruses isolated from this patient were more closely related to each other than to any other virus in the analysis. Notably, GN285 and ON129 clustered together in 376 of the 500 bootstrap tests.

Conclusions

Our data indicate that oseltamivir resistance developed within 2 days. This time is similar to the interval for development of resistance to adamantanes in subtype H3N2 viruses when 30% of treated patients shed resistant, trans-

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missible virus within 3 days of beginning treatment (7). Four cases of H275Y infection have been detected, with the use of sequencing, among 1,060 pandemic (H1N1) 2009 isolates tested (0.47%) in Singapore since June 2009 (not including the case reported here). Only 1 case was found in a pretreatment sample; the other 3 were identified after

treatment. The emergence of H275Y might also be affected by the timing of therapy. In the case reported here, oseltamivir treatment was begun on day 4 of illness when viral titers were almost maximal, which is probably the stage of illness best suited to select for resistant mutants because the presence of mutations is likely to be greatest when replica-

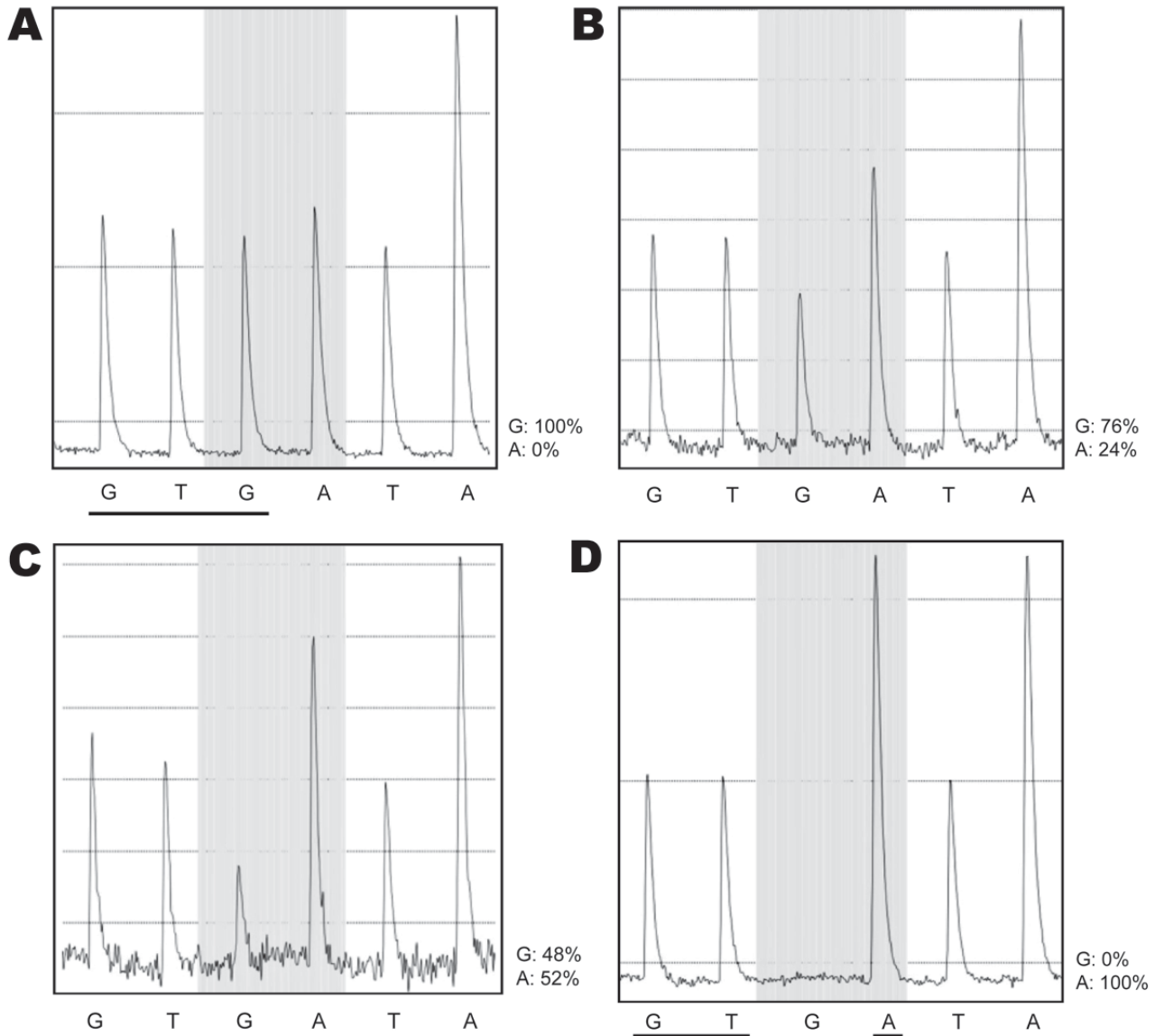


Figure. Pyrograms showing evolution of the H275Y mutation in pandemic (H1N1) 2009 virus, Singapore. A) May 29, sample 14 h after receiving oseltamivir shows 100% G. B) May 30, 38-h sample shows 76% G and 24% A. C) May 30, sample at 45 h shows 48% G and 52% A. D) May 30, virus isolated from 38-h sample is 100% A. The shaded area indicates the mutation site, showing the progressive loss of the third base, G, and its replacement by A. In panel A, all bases have peaks of equivalent height because each base is a singlet, except for the last peak, which is double the height and represents an A followed by another A, as in AA. In panel D, the G at the mutation site has disappeared and the signal of the next base, A, has doubled in amplitude, indicating the complete replacement of G by A. This reflects replacement of the complementary base C by T in the viral template. The 3 bases that constitute aa 275 in the neuraminidase protein are underlined in panels A and D. In panel A, the sequence is 100% GTGAATA. In panel D, it is 100% GTAAATA. In panel A, the wild-type 5'-GTG-3' is equivalent to 3'-CAC-5' in its plus-strand RNA, which codes for histidine (H). Similarly, the mutant 5'-GTA-3' in panel D is equivalent to 3'-CAT-5', which codes for tyrosine (Y). Therefore, the pyrograms show a mutation from H to Y at position 275—the H275Y mutant.

tion is greatest. Notably, rates of H275Y are high, reaching 13% (8) among immunocompromised groups in whom high viral titers might also be a contributory factor.

Pyrosequencing directly on clinical material allows the measurement of relative quantities of viral variants without introducing errors inherent in viral culture, which is known to favor the growth of H275Y mutants (6). Pyrosequencing cannot exclude the presence of subpopulations of <5%–10%, but if small proportions of mutant virus had been present, they would have been detected in the culture of the sample collected 14 hours after exposure to oseltamivir; however, only wild-type sequences were detected. Similarly, only wild-type sequences were detected in 192 clones derived directly from the sample collected a few hours before the first dose of oseltamivir. The phylogenetic data show, at the amino acid and nucleotide level, that the resistant and sensitive isolates cluster together, apart from other viruses. These data support the hypothesis that the H275Y mutant arose de novo from the wild-type virus from the same patient.

The fact that the effects of oseltamivir are likely to be greatest in severe disease (9–11), but of modest benefit in mild infections (12), has led to proposals for restricting the use of antiviral agents and the use of alternative antiviral agents and multidrug therapy to prevent the emergence of resistance (7,9) in severe cases. The proposed interventions may be of little consequence compared with the association and co-selection of H275Y with other genetic determinants. Although in isolation H275Y compromises seasonal influenza (H1N1) by reducing the amount of neuraminidase expressed on the cell surface, other mutations (R194G, V234M, and R222Q) may compensate and restore its expression to levels found in wild-type virus, without H275Y (13). This circumstance may explain the emergence and spread of H275Y in the absence of drug pressure in seasonal influenza (H1N1), which increased from being negligible in 2007 to 95% in March 2009 (7) despite a low consumption of oseltamivir (14). More than 99% of all pandemic (H1N1) 2009 neuraminidases have G at position 194, which corresponds to the R194G in seasonal influenza (H1N1). However, the effects of mutations are not easily transferable among different influenza (H1N1) types and may need to be tested separately (online Technical Appendix).

The clinical effects of the spread of H275Y in seasonal influenza (H1N1) have been minimal because seasonal influenza (H1N1) now accounts for an insignificant proportion of influenza (15). However, if H275Y mutants of pandemic (H1N1) 2009 emulate the expansion of resistant seasonal influenza (H1N1), the effect might be substantial because pandemic (H1N1) 2009 accounted for 90%–95% of circulating influenza A viruses in the Northern and Southern Hemispheres in late 2009 (15). We are speculat-

ing, however, because our laboratory data show that pandemic (H1N1) 2009 fell from 62% to 29% of 436 influenza cases from May 2010 to mid June 2010, Singapore's main influenza season, whereas the presence of influenza (H3N2) has risen from 23% to 53% (influenza B accounts for 15%–20%). As the next influenza season in the Southern Hemisphere approaches, the relative mixture of subtype H3N2 and H1N1 viruses will be under scrutiny again, not only to predict "best bet" vaccine components but also to ascertain their associated resistance patterns. Whatever the epidemiologic data exhibit, clinicians should consider resistance when patients do not respond to treatment for pandemic (H1N1) 2009 because H275Y can emerge literally overnight, as the case reported here reminds us.

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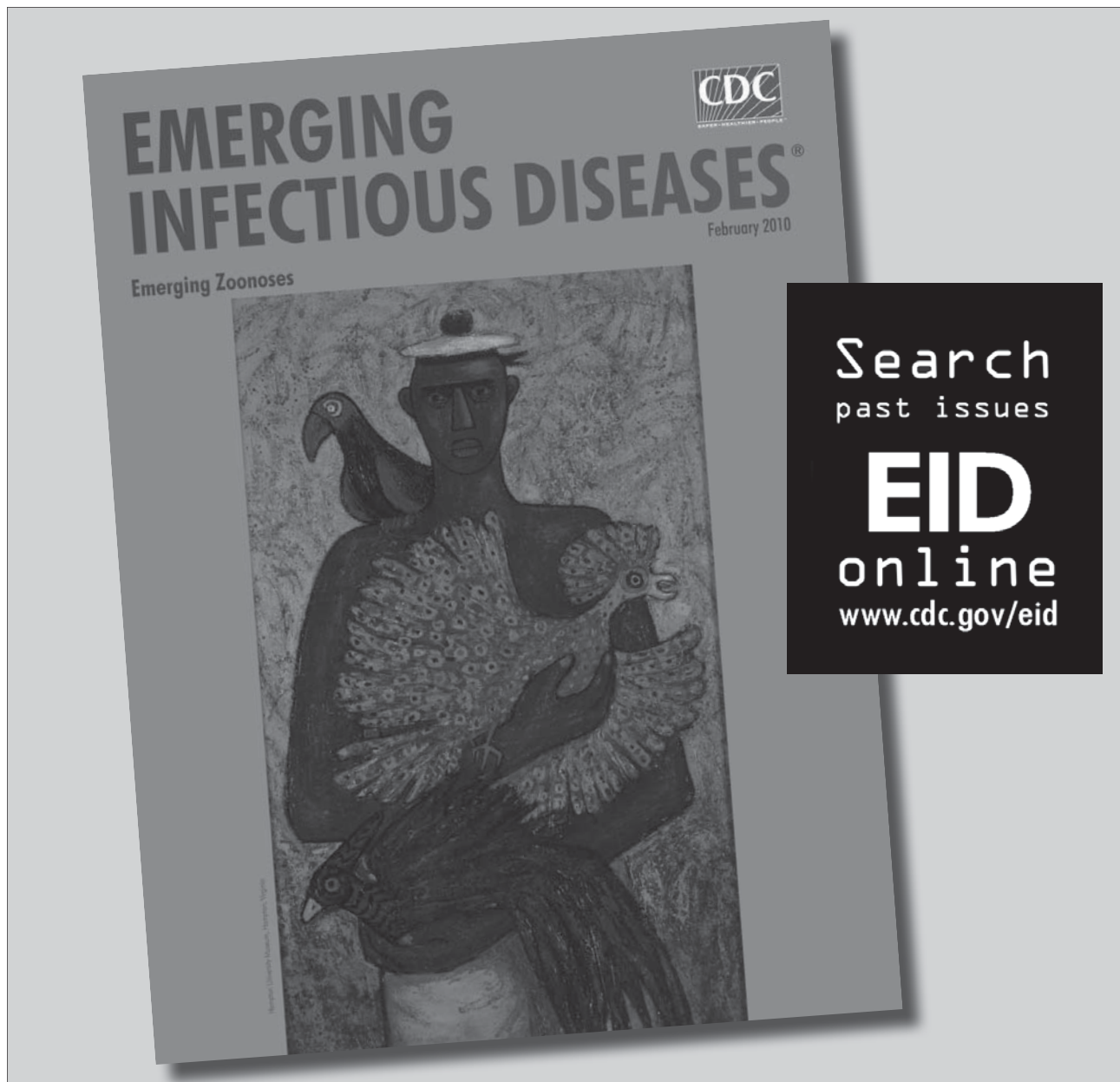
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Emergence of Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus Within 48 Hours

Technical Appendix

Experimental Methods

Clinical data and daily combined nasal and throat swab specimens were collected prospectively in universal transport medium. Nucleic acids were extracted by using easyMag (bioMérieux, Marcy-l’Etoile, France); aliquots were frozen at -80°C . Real-time reverse transcription-PCR (RT-PCR) for influenza A (H1N1) 2009 was carried out with an in-house assay on an Mx3005P instrument (Stratagene, La Jolla, CA, USA). Virus isolation was carried out with Madin-Darby canine kidney cells. RNA extracted directly from samples and from culture supernatants was sequenced. The full genome of the wild-type isolate, A/Singapore/ON129/2009(H1N1), was sequenced, according to published methods (1) and, among the 8 segments, the nucleoprotein and RNA polymerase B segments were resequenced with a protocol from the World Health Organization Collaborating Center for Influenza at the Centers for Disease Control and Prevention (Atlanta, GA, USA) (http://www.who.int/csr/resources/publications/swineflu/pyrosequencing_protocol/en/index.html). Their GenBank accession nos. are CY049065, CY049066, CY049067, CY049068, CY049069, CY049070, CY049071, and CY049072. The resistant isolate /Singapore/GN285/2009(H1N1) was sequenced with the same Centers for Disease Control and Prevention protocol. The GenBank accession nos. are CY055300, CY055301, CY055302, CY055303, CY055304, CY055305, CY055306, and CY055307. A PCR fragment ≈ 1 kb (obtained directly, without culture, from the clinical sample collected a few hours before initiation of oseltamivir on day 4 of illness) containing the aa position 275 on neuraminidase (NA) (the mutation point) was cloned into a pGEM-T vector (Promega Corp., Madison, WI, USA), and 192 clones picked and sequenced.

Creation of Amplicons for Pyrosequencing

RT-PCR was performed with the SuperScript III One-Step RT-PCR System with Platinum Taq (cat. no: 12574-018; Invitrogen Life Technologies, Carlsbad, CA, USA) in a 50- μ L reaction volume containing 5 μ L of RNA sample. The biotinylated PCR product was generated with forward primer, (5'-Bio/TGCTTTACTGTAATGACCGAT-3') at a final concentration of 0.25 μ mol/L and reverse primer, (5'-GATTCTGGTTGAAAGACACCC-3') at a final concentration of 0.2 μ mol/L in a thermal cycler. The resulting 217-bp amplicon accommodates the codon for H275 (CAC) or H275Y (TAC) in the product. A Mastercycler-ep-gradient-S (Eppendorf, Hamburg, Germany) was used with the following steps and conditions: reverse transcription at 55°C for 10 min and initial denaturation at 94°C for 2.5 min; followed by 40 cycles of denaturation at 94°C for 32 s, annealing at 57°C for 76 s, and extension at 68°C for 33 s; and a final extension at 68°C for 5 min. PCR products were analyzed by gel electrophoresis to estimate the yield.

Pyrosequencing

Pyrosequencing was performed according to the manufacturer's guidelines (Biotage, Uppsala, Sweden). Briefly, \approx 200 ng of biotinylated PCR product was reacted with streptavidin-coated beads (GE Healthcare, Little Chalfont, UK) by shaking at room temperature for 15 min, followed by collecting DNA-coated beads by vacuum onto a 96-well vacuum tool and serially immersing the beads into 70% ethanol, 0.2 mol/L NaOH, and 10 mmol/L Tris-acetate, pH 7.6, washing buffer. The beads with single-stranded DNA template were released into each well of PSQ96-well plate (Biotage) with 40 μ L of annealing buffer (20 mmol/L Tris-acetate, pH 7.6, and 2 mmol/L magnesium acetate) containing the sequencing primer (5'-TAGAATCAGGATAACAGGAGCA-3') at a final concentration of 0.4 μ mol/L. The plate was heated at 80°C for 2 min and then cooled to room temperature for 10 min before being placed into the PyroMark Q96 ID System (Biotage). The sequencing procedure was performed at room temperature by cyclic dispensation of substrates, enzymes, and 4 dNTPs (Biotage) in a prespecified order to enable single nucleotide polymorphism analysis and generation of quantitative data. We sequenced a 25-bp region that included the H275Y mutation of NA. Relative proportions of bases, expressed as a percentage, were determined by using the PyroMark instrument.

The description of the mutation as H275Y means that the mutation in the gene results in the original amino acid at position 275 along NA (in N1 numbering) changing from the expected wild-type histidine (H) to tyrosine (Y). The actual change in the NA gene is a single point mutation from a cytosine base to a thymidine base.

Phylogenetic Analysis

All 7 strains with the NA H275Y mutation for which the whole genome (10 genes from 8 segments) was available from the National Center for Biotechnology Information (Bethesda, MD, USA) influenza virus resource (2) were compared. A drug-sensitive isolate (ON141), which was geographically and temporally closely related (a difference of <1 week) with full genome available, was also included. Because the nucleotide level also enables seeing synonymous nucleotide exchanges (without an amino acid change) that could harbor additional similarity information, we chose to derive a maximum likelihood tree over the whole coding genome to investigate the relationship of these 10 strains in further detail. Nucleotide alignments of the coding regions of all 10 genes in the 8 segments of the 10 strains under study were concatenated. A maximum likelihood tree was calculated with PhyML3 (3) by using the following parameters: tree search = NNIs; initial tree = BIONJ; model of nucleotides substitution = HKY85; log-likelihood = -18939.44126; discrete gamma model = yes, number of categories = 4, gamma shape parameter = 97.954 (estimated by PhyML); proportion of invariant = 0.923, transition/transversion ratio = 16.943; bootstrap steps = 500. The final tree was displayed and exported from MEGA4 (4).

Technical Appendix Table 1. Results for 6 respiratory samples collected from patient infected with pandemic (H1N1) 2009 over 4 days, Singapore*

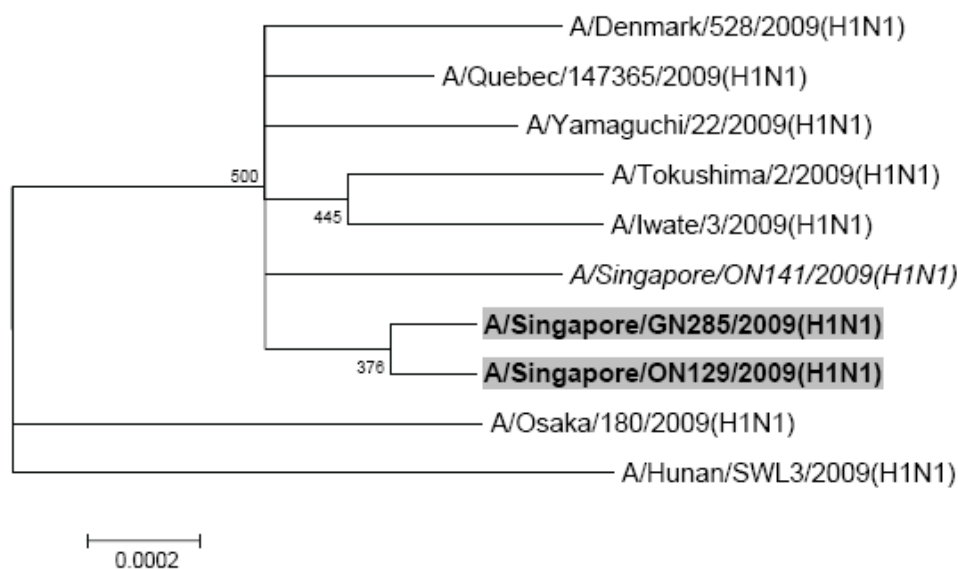
| Characteristic | | | | | | |
|--|------|------|----------|----------|----------|---------|
| Day of illness | 3 | 3 | 4 | 5 | 6 | 6 |
| Sample date in May 2009 | 27 | 27 | 28 | 29 | 30 | 30 |
| Hours after oseltamivir initiated | – | – | 0 | 14 | 38 | 45 |
| Oseltamivir, 75 mg | None | None | 1 dose | 2 doses | 2 doses | 2 doses |
| RT PCR, C _t value | 32 | 33 | 29 | 24 | 32 | 35 |
| Pyrosequencing directly on sample, % | 100 | 100 | 100 | 100 | 76 | 48 |
| Wild type | | | | | | |
| Mutant | 0 | 0 | 0 | 0 | 24 | 52 |
| Sequencing of 192 cloned fragments obtained directly from the clinical sample, % | – | – | 100 | – | – | – |
| Wild-type | | | | | | |
| Mutant | – | – | 0 | – | – | – |
| BigDye sequencing directly on clinical sample, % | – | – | 100 | – | – | – |
| Wild type | | | | | | |
| Mutant | – | – | 0% | – | – | – |
| Viral isolation | – | – | Positive | Positive | Positive | – |
| BigDye sequencing of isolated virus, % | – | – | 100 | 100 | 0 | – |
| Wild-type | | | | | | |
| Mutant | – | – | 0 | 0 | 100 | – |
| Pyrosequence of isolated virus, % | – | – | 100 | 100 | 0 | – |
| Wild type | | | | | | |
| Mutant | – | – | 0 | 0 | 100 | – |

*RT-PCR, reverse transcription PCR for Influenza A (H1N1) 2009; C_t, cycle threshold; mutant, H275Y mutation –, not done/not applicable; .

Technical Appendix Table 2. Amino acid differences (mutations) between the 10 viral isolates included in the phylogenetic analysis compared with the early reference strain A/Texas/05/2009(H1N1)*

| Strain | Protein | | | | | | | | | |
|-------------------------------|---------------------|-------|-------------|--------------|-------------|---------------------|-------|----|-------------------|------|
| | PB2 | PB1 | PA | HA | NP | NA | M1 | M2 | NS1 | NS2 |
| A/Iwate/3/2009 (H1N1) | | | | S220T | V100I | V106I, N248D, H275Y | K113R | | I123V | |
| A/Yamaguchi/22/2009 (H1N1) | | | V379I | S220T | V100I | V106I, N248D, H275Y | | | I123V | |
| A/Hunan/SWL3/2009 (H1N1) | S155N, M243L, I398V | | T357A | | K90R, V100I | V106I, N248D, H275Y | | | N48K, V60I, D101E | |
| A/Quebec/147365/2009 (H1N1) | | | A20T, L686V | S220T, A273S | V100I | V106I, N248D, H275Y | | | M79T, I123V | |
| A/Denmark/528/2009 (H1N1) | | F730S | | S220T | V100I | V106I, N248D, H275Y | | | M93I, I123V | |
| A/Tokushima/2/2009 (H1N1) | | | M311I | S220T | V100I | V106I, N248D, H275Y | | | I123V | |
| A/Osaka/180/2009 (H1N1) | V649I, E700K | I667T | | V169I | V100I | V106I, H275Y | A33T | | | E63K |
| A/Singapore/GN285/2009 (H1N1) | | I435V | | S220T | V100I | V106I, N248D, H275Y | | | I123V | |
| A/Singapore/ON129/2009 (H1N1) | | I435V | | S220T | V100I | V106I, N248D | | | I123V | |
| A/Singapore/ON141/2009 (H1N1) | K482R | | | S220T, R238G | V100I | A86V, V106I, N248D | | | I123V | |

* PB1, PB2, polymerase basic 1 and 2; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M1, M2, matrix 1 and 2; NS1, NS2, nonstructural 1 and 2.



Technical Appendix Figure. Maximum-likelihood phylogenetic tree. Shaded strains are oseltamivir susceptible (Singapore/ON129) and resistant (Singapore/GN285) isolates from the case presented. Singapore/ON141 is a drug-sensitive isolate geographically and temporally closely related to ON129 and GN285 is shown in italics. The remaining strains in the tree are all isolates with the H275Y mutation. Scale bar indicates nucleotide substitutions per site.

Effects of Permissive Mutations

A/Singapore/GN285/2009(H1N1) and 99.9% of all pandemic (H1N1) 2009 NAs have G at position 194, which would correspond to the R194G mutation that resulted in rescue of the expression deficit caused by the H274/275Y mutation in the context of the WSN laboratory strain. However, as shown in Figure 2B in the article by Bloom et al. (5), there remains an expression deficit with the H274/275Y mutation in the context of A/California/04/2009(H1N1), which also has G at position 194. Consequently, the results and effects of the respective mutations are not easily transferable among different H1N1 subtypes and may need to be tested separately.

For completeness, the most typical amino acid at position 222 among the pandemic NAs is N, which is physicochemically similar to the Q of the R222Q mutation. However, the 2 preceding residues also differ between seasonal and pandemic NAs, which may alter the local structure and consequently potential effects on other positions. At position 234, most pandemic

(H1N1) 2009 sequences (including those of A/Singapore/GN285/2009[H1N1] and A/California/04/2009[H1N1]) have the nonpermissive V. Effects of the NA mutations V106I and N248D on expression of the virus are not known at present, but because of their common occurrence in later phases of the pandemic (thus far), they should not be considered unfavorable for the virus.

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