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# A comparison of pyrosequencing and neuraminidase inhibition assays for the detection of oseltamivir-resistant pandemic influenza A(H1N1) 2009 viruses

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ABSTRACT

Currently most pandemic influenza A(H1N1) 2009 (H1N1pdm) viruses are sensitive to oseltamivir, but a single point mutation (H275Y) in the neuraminidase (NA) gene of H1N1pdm can lead to resistance and such viruses have been reported from several countries. In this study we compare the performance of a pyrosequencing-based method for the detection of the H275Y mutation in H1N1pdm viruses with a conventional NA inhibition assay. Pyrosequencing could detect as little as 5% H275Y mutants in a mixed viral population, while mixtures with 25% or greater mutant virus were required before a significant increase in $IC_{50}$ value could be detected. However, the sensitivity of the NA inhibition assay could be enhanced by using a more sophisticated curve-fitting analysis to generate similar results to the pyrosequencing assay. Of 181 H1N1pdm clinical samples examined by pyrosequencing, nine samples from five patients were found to contain H275Y mutant viruses, four of whom were under oseltamivir treatment. Changes in the ratio of H275Y mutant to wild-type viruses were observed in serial clinical specimens from two patients over the duration of their treatment. This study highlights the need for close monitoring of the H275Y mutation in clinical samples, in particular from severely ill patients infected with H1N1pdm. The use of pyrosequencing and the NA inhibition assay provide powerful tools for the rapid detection and quantitation of resistant influenza viruses in mixed populations.



## 1. Introduction

The neuraminidase inhibitor (NAI) antiviral drugs oseltamivir (Tamiflu™) and zanamivir (Relenza™) have been widely used to treat and prevent infection with the pandemic influenza A(H1N1) 2009 (H1N1pdm) virus since it emerged in March–April 2009 (Uyeki, 2009; WHO, 2009). To date, most of the circulating H1N1pdm viruses tested remain sensitive to oseltamivir. However, cases of resistance to oseltamivir caused by a single amino acid change, histidine to tyrosine at position 275 (H275Y; N1 numbering) in the neuraminidase (NA) (Ives et al., 2002), have been detected in various regions of the world, with WHO reporting 314 oseltamivir-resistant viruses up to 1 December 2010 (WHO, 2010). The H275Y mutation causes high level resistance to oseltamivir, but has no effect on zanamivir susceptibility (Gaur et al., 2010).

To date oseltamivir resistance has mainly been detected in immunocompromised patients and in individuals under treatment or prophylaxis with oseltamivir (CDC, 2009; Harvala et al., 2010; WHO, 2010), with little onward transmission of oseltamivir-resistant viruses (Gulland, 2009; Harvala et al., 2010; Le et al., 2010) mostly in hospital settings (Gulland, 2009) or following prolonged exposure (Baz et al., 2009).

The susceptibility of influenza viruses to the NAIs has traditionally been determined by the NA inhibition assay. While this is a simple high-throughput phenotypic assay, it requires cultured viruses and cannot be applied directly to original clinical samples (Hurt et al., 2004). Alternatively, NAI resistance can be identified in viruses from original specimens by conventional sequencing for mutations known to confer reduced NAI susceptibility, but this is

*Abbreviations:* RT-PCR, reverse transcriptase polymerase chain reaction; WHO, World Health Organisation; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; HA, hemagglutinin; NA, neuraminidase; H275Y, histidine to tyrosine substitution at position 275; NAI, neuraminidase inhibitor; H1N1pdm, pandemic influenza A(H1N1) 2009; ICU, intensive care unit; MDCK, Madin-Darby canine kidney; SD, standard deviation; LDR, log dose–response.

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**Table 1**
Primer sequences designed for RT-PCR and pyrosequencing.

| Primer | Sequence 5′–3′ | Gene | Assay |
|---|---|---|---|
| pdmHAI-F[a] | TGTAAAACGACGGCCAGTATGAAGGCAATACTAGTAG | HA | RT-PCR |
| pdmHAI-R[b] | CAGGAAACAGCTATGACCGATCGGATGTATATTCTGAAATGG | HA | RT-PCR |
| pdmHAII-F[a] | TGTAAAACGACGGCCAGTGATTGCAATACAACTTGTC | HA | RT-PCR |
| pdmHAII-R[b] | CAGGAAACAGCTATGACCTACACTGTAGAGACCCATTAGAGC | HA | RT-PCR |
| pdmNAI-F[a] | TGTAAAACGACGGCCAGTATGAATCCAAAYCARAAGAT | NA | RT-PCR |
| pdmNAI-R[b] | CAGGAAACAGCTATGACCGTGATAATTAGGGGCATTC | NA | RT-PCR |
| pdmNAII-F[a] | TGTAAAACGACGGCCAGTGACAGGCCTCATACAAGATCTTC | NA | RT-PCR |
| pdmNAII-F[b] | CAGGAAACAGCTATGACCTAAATGGMAACTCAGCACCG | NA | RT-PCR |
| PypdmNA-R[c] | Biotin-GACAGGCCTCATACAAGATCTTC | NA | Pyrosequencing |
| PyN1NA-R | TGCCAGTTRTCCCTGCAYACACA | NA | Pyrosequencing |
| PypdmNA-S[d] | TAACAGGAGCATTCCTCATA | NA | Pyrosequencing |

[a] Primers with M13 forward sequence attached to the 5′ end.
[b] Primers with M13 reverse sequence attached to the 5′ end.
[c] Primer was biotinylated at the 5′ end.
[d] Sequencing primer for pyrosequencing.

time-consuming and not high throughput. Recently, we and others have used pyrosequencing to detect oseltamivir-resistant mutants among seasonal H1N1 and H1N1pdm 2009 viruses (Deyde et al., 2010; Dharan et al., 2009; Hurt et al., 2009a; Lackenby et al., 2008a). This method is rapid and high throughput, and can be applied to original clinical samples or influenza virus isolates. In this study, we further compared pyrosequencing for quantitating H275Y mutants in viral mixtures containing both oseltamivir resistant and wild-type viruses and the NA inhibition assay. We also demonstrated that the ability of the NA inhibition assay to predict the percentage of oseltamivir resistance viruses in a mixed viral population was significantly improved when combined with a novel curve-fitting analysis.

## 2. Materials and methods

### 2.1. Virus strains and clinical specimens

A total of 181 clinical specimens from 129 patients confirmed to be H1N1pdm positive by real-time RT-PCR were collected (Chidlow et al., 2010), including 50 specimens from 50 community outpatients, 50 from hospitalized patients, 45 from 13 intensive care unit (ICU) patients, and 36 from 16 deceased patients. Viruses were grown in Madin-Darby canine kidney (MDCK) cells (Barr et al., 2003), A/Auckland/1/2009 and A/Osaka/180/2009 were used as reference wild-type and H275Y mutant H1N1pdm viruses, respectively.

### 2.2. RNA extraction, real-time RT-PCR and RT-PCR

Viral RNAs were extracted from clinical specimens or cultured viruses (Barr et al., 2008). Superscript III one-step qRT-PCR kit (Invitrogen, Carlsbad, CA) was used for real-time RT-PCR with influenza A matrix specific primers and probes (Dawood et al., 2009) on an ABI 7500Fast instrument (Applied Biosystems, Foster City, CA). Superscript III one-step RT-PCR kit (Invitrogen) was used for RT-PCR with primers listed in Table 1.

### 2.3. Pyrosequencing

Single-stranded biotinylated DNA from the 145-bp PCR product flanking the H275Y mutation site of the NA gene was purified using the PyroMark Vacuum Prep Workstation (Qiagen, Valencia, CA) according to the manufacturer's recommendation. Pyrosequencing reactions were performed on a PyroMark ID (Qiagen) with the PyroMark Gold reagents (Qiagen), and results were analysed using the AQ mode in the PyroMark ID 1.0 software to estimate the percentage of H275Y mutant and wild-type by calculating the ratio of the two peaks representing the wild-type and mutant viruses.

### 2.4. Neuraminidase enzyme inhibition assay

Oseltamivir carboxylate, the active form of the ethyl ester prodrug oseltamivir phosphate (Hoffmann-La Roche Ltd., Switzerland) and zanamivir (GlaxoSmithKline, Australia) were used for the NA inhibition assay as previously published (Hurt et al., 2004). The concentrations required to inhibit 50% of NA activity ($IC_{50}$) were calculated using a logistic curve fit program "Robosage" (GlaxoSmithKline, UK).

### 2.5. Curve-fitting analysis

An investigational curve-fitting analysis was used to estimate the H275Y mutant proportion in a mixed population by fitting three parameter logistic dose–response (LDR) functions to the inhibition data for 100% H275Y mutant and 100% wild-type viruses using TableCurve2D (SPSS Software, Chicago, IL). The model was generated based on the assumption that the total inhibitory effect on mixed populations would be the sum of the individual effects on the component populations so that $y = \%$ measured inhibition $= [(1-\alpha)(\%\text{inhibition of wt}) + (\alpha)(\%\text{ inhibition of mutant})]$ where $\alpha$ is the proportion of mutant H275Y virus present. TableCurve2D was used to fit the model to each set of NAI data in order to estimate $\alpha$.

## 3. Results

### 3.1. Detection of H275Y mutant virus in a mixed population by pyrosequencing and NA inhibition assays

Preliminary experiments established the protocols for distinguishing and quantifying mixture of wild-type and oseltamivir-resistant mutant H275Y NA sequences by pyrosequencing (data not shown). Mixtures of wild-type and mutant H275Y viral isolates were prepared based on their viral copy number determined by real-time RT-PCR, and were used to compare the ability of pyrosequencing and the NA inhibition assay to detect low proportions of oseltamivir-resistant mutant viruses in a mixed population (Table 2). Pyrosequencing could detect as little as 5% H275Y mutant virus in the mixture. In comparison, mixtures containing at least 25% mutant virus were required before a significant increase in $IC_{50}$ value was detected in the NA inhibition assay (Fig. 1). However, a more sophisticated analysis of the inhibition data for the 100% H275Y mutant and 100% wild-type viruses allowed a model to be formulated and fitted to the inhibition curves of the various mixed

**Table 2**
Limit of detection of H275Y mutant viruses in mixed populations by pyrosequencing and NA inhibition assays.[a]

| % Mutant | NA inhibition assay | | Pyrosequencing assay |
|---|---|---|---|
| | Mean $IC_{50}$ (nM) ± SD | LDR model[b] estimate of mean %H275Y ± SD | Mean %H275Y ± SD |
| 100 | 414.2 ± 55.5 | 97.2 ± 2.2 | 95.6 ± 4.4 |
| 75 | 330.4 ± 32.8 | 71.5 ± 2.1 | 85.0 ± 4.9 |
| 50 | 134.8 ± 29.8 | 46.9 ± 1.8 | 55.5 ± 5.0 |
| 25 | 5.4 ± 3.5 | 23.4 ± 1.1 | 35.5 ± 3.7 |
| 20 | 1.0 ± 0.3 | 18.4 ± 1.1 | 21.8 ± 4.9 |
| 10 | 0.7 ± 0.2 | 10.8 ± 0.6 | 13.4 ± 3.6 |
| 7.5 | 0.5 ± 0.2 | 7.2 ± 1.2 | 7.9 ± 3.3 |
| 5 | 0.6 ± 0.2 | 6.9 ± 3.0 | 7.3 ± 2.6 |
| 0 | 0.6 ± 0.2 | 0.0 ± 1.5 | -0.5 ± 3.6 |

[a] The virus copy numbers of wild-type and mutant viruses were determined separately by real-time RT-PCR. The viruses were then mixed in the indicated proportions before testing in triplicate in the two assays.
[b] The individual LDR equations for 100% H275Y mutant and 100% wild-type viruses were used to generate the model: $y$ = % measured inhibition = [(1 − $\alpha$)(%inhibition of wt) + ($\alpha$)(% inhibition of mutant)] where $\alpha$ is the proportion of mutant H275Y in the viral population, and TableCurve2D was used to fit the model to inhibition data and estimate the % of H275Y present.

populations, so that an estimate of the proportion of H275Y mutant could be calculated. Interestingly, the estimates for the mixed populations derived from NA inhibition assay model were generally similar to both the pyrosequencing and expected values for the different mixtures (Table 2).

### 3.2. Oseltamivir-resistant viruses were detected in five patients undergoing oseltamivir treatment

The pyrosequencing assay was used to monitor the prevalence of H275Y mutant viruses in 181 clinical specimens from 129 patients infected with H1N1pdm viruses. H275Y mutant viruses were detected at levels varying from 5 to 94% in specimens from five patients in hospital or ICU, of which four were under oseltamivir treatment (Table 3). Virus isolation was attempted on all 14 specimens of the five patients, but virus isolates were only obtained from four specimens, two of these were pure wild-type viruses, and the other two were a mixture of wild-type and mutant viruses. NA inhibition assays performed on the four virus isolates confirmed their susceptibility to oseltamivir (Table 3). For patient No. 4, the percentage of H275Y mutant in the mixture rose from 17% in the original specimen to 89% in the virus isolates following two passages in MDCK cells (Table 3). Pyrosequencing analysis of the specimens from 50 community patients revealed that none contained viruses with the H275Y mutation.

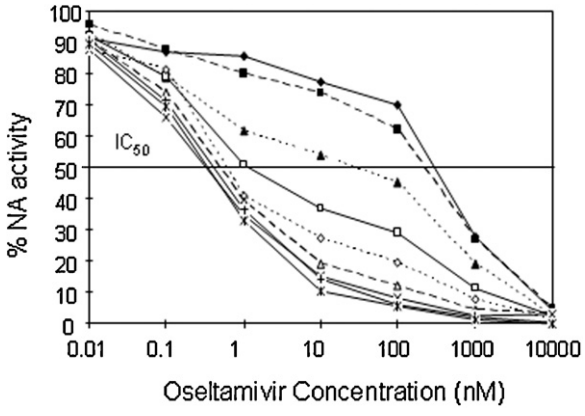


**Fig. 1.** NA inhibition assay results for mixed H275Y mutant and wild-type viruses. H275Y mutant and wild-type viruses were mixed in different ratios in triplicate to give 100% (♦), 75% (■), 50% (▲), 25% (□), 20% (◊), 10% (△), 7.5% (×), 5% (+), and 0% (*) of the H275Y mutant virus. NA activities of the mixtures were measured in the presence of oseltamivir at concentrations indicated in log scale on the $x$-axis.

### 3.3. Confirmation of de novo mutation of the oseltamivir-resistant viruses from two patients

Pyrosequencing analysis of serial specimens from two patients identified the H275Y mutant at varying proportions in samples collected on different dates during oseltamivir treatment (Table 3). Only wild-type viruses were detected in the nasal wash sample of patient 1 on Day 0, but following oseltamivir treatment, H275Y mutants (in the range of 73–94%) were detected in sputum, nasal wash and bronchial washing samples collected on days 15–29. On Day 31, the H275Y mutant became undetectable and total viral load also dropped significantly to less than 100 viral RNA copies/μl of specimen. Only two specimens from patient 1 yielded isolates in MDCK cells and these were used for the NA inhibition assay. The results confirmed that, while the Day 0 sample was sensitive to oseltamivir ($IC_{50}$ = 0.3 ± 0.1 nM), the sensitivity of the Day 19 sample was reduced by >1000 fold ($IC_{50}$ = 296.7± 20.0 nM) (Table 3). For patient 2, although only wild-type viruses were detected by pyrosequencing on Day 1, 34% and 10% H275Y mutants emerged on Day 11 and 17, respectively, however none of the virus from these samples could be cultured for the NA inhibition assay.

As the two patients were treated in the same ICU at around the same time, the HA and NA genes from oseltamivir-sensitive and resistant specimens of both patients were sequenced to determine their similarity. The sequences obtained from the sensitive viruses of the two patients differed by 3 nucleotides in HA and 1 in NA, none of which led to an amino acid substitution. Within each patient, the HA and NA sequences of the sensitive and resistant viruses were identical apart from the H275Y mutation (Table 4). This suggests that these cases were independent and the oseltamivir-resistant viruses emerged as a result of drug pressure, rather than by transmission from one ICU patient to the other.

## 4. Discussion

Currently, assays that can be used to detect oseltamivir resistant viruses include the traditional NA enzyme inhibition assay, Sanger sequencing and allele-specific real-time RT-PCR and pyrosequencing. The pyrosequencing method described here and elsewhere (Deyde et al., 2010; Dharan et al., 2009; Hurt et al., 2009a; Lackenby et al., 2008a) can detect the oseltamivir resistance mutation in both original clinical (containing either viable or nonviable viruses) specimens and cultured viruses, unlike the traditional NA enzyme inhibition assays that can only be performed on cultured viruses. This is important because it is not always possible to culture viruses from clinical samples, especially those undergoing antiviral drug treatment, as was the case in this study where from 14 clinical

**Table 3**
Characteristics of patients with viruses containing the H275Y mutation.

| Patient details | | | | Real-time RT-PCR Ct value | Specimen date since Oseltamivir treatment | % H275Y[a] | | Mean $IC_{50} \pm SD$ (nM)[b] |
|---|---|---|---|---|---|---|---|---|
| | Immune Status | Ward type | Specimen type | | | Specimen | Isolate | Isolate |
| 1 | Compromised (renal transplant) | ICU | Nose/throat swab | 32 | Day 0 | 0 | 0[c] | $0.3 \pm 0.1$[c] |
| | | | Sputum | 24 | Day 15 | 91 | – | – |
| | | | Bronchial washing | 25 | Day 19 | 81 | 89[d] | $296.7 \pm 20.0$[d] |
| | | | Nose/throat swab | 31 | Day 20 | 80 | – | – |
| | | | Nose/throat swab | 36 | Day 29 | 94 | – | – |
| | | | Sputum | 34 | Day 31 | 0 | – | – |
| 2 | Competent | ICU | Nose/throat swab | 23 | Day 1 | 0 | 0[d] | $0.3 \pm 0.1$[d] |
| | | | Sputum | 24 | Day 11 | 34 | – | – |
| | | | Nose/throat swab | 26 | Day 17 | 10 | – | – |
| 3 | Competent | ICU | Nose/throat swab | 30 | Day 0 | 0 | – | – |
| | | | Sputum | 32 | Day 3 | 5 | – | – |
| | | | Nose/throat swab | 32 | Day 8 | 0 | – | – |
| 4 | Compromised (Chemotherapy for cancer) | Hospital | Sputum | 31 | Day 3 | 17 | 89[c] | $306.7 \pm 21.2$[c] |
| 5 | Competent | Hospital | Nose/throat swab | 20 | Day 0 | 6 | – | – |

[a] Pyrosequencing was performed on clinical specimens and/or virus isolates grown from original specimens to determine the percentage of H275Y mutant viruses.
[b] Mean $IC_{50} \pm SD$ of 3 replicates was determined by NA inhibition assay of virus isolates cultured from original specimens. The $IC_{50}$ of the oseltamivir-sensitive wild-type virus was $0.39 \pm 0.2$ ($n = 3$).
[c] Virus isolate obtained after two passages of MDCK cell culture.
[d] Virus isolate obtained after one passage of MDCK cell culture.

specimens from patients undergoing oseltamivir treatment that were positive for H1N1pdm by real-time RT-PCR, only four could be isolated in cell culture. In addition, growth of viruses in cell culture may alter the proportion of resistant virus in the original clinical sample, especially if a number of passages in MDCK cells are required (Hurt et al., 2009b; Okomo-Adhiambo et al., 2010), as was seen in patient No. 4 in our study, where a significant rise in the percentage of the H275Y mutant was observed following two passages in MDCK cells (17–89% in Table 3). Pyrosequencing was highly sensitive and capable of rapid high-throughput analysis (taking less than 5 h to obtain a result for up to 96 viruses). Pyrosequencing could also detect mixtures of as little as 5% H275Y virus mutants with wild-type virus. Alternative H275Y mutation detection assays that use real-time RT-PCR allelic discrimination (Chidlow et al., 2010; Renaud et al., 2010; van der Vries et al., 2009), are sensitive in detecting pure H275Y mutants or wild-type viruses, but their ability to quantify levels of H275Y mutants in a viral mixture is limited, particularly if there are other mismatches in either the primer or probe binding sequences. This is because these mismatches can decrease the PCR efficiency and the results are often indistinguishable from that produced from a mixture containing both wild-type and mutant viruses (unpublished observation).

The ability of the NA inhibition assay to detect low proportions of H275Y mutant in a mixed population was significantly improved by the use of a novel curve fitting method. This is important because it improves the detection limit commonly seen with $IC_{50}$ determination where at least 25% of the H275Y mutant was required in a mixed viral population before a significant difference from the wild-type $IC_{50}$ could be determined.

The presence of low levels of oseltamivir-resistant mutants may not be clinically significant for most immunocompetent patients because the majority of wild-type viruses in the viral population are still sensitive to oseltamivir, and the immune system should clear the remaining oseltamivir-resistant viruses. However, oseltamivir-resistant viruses may have a more significant impact on immunocompromised patients, as they tend to shed virus for a longer period (Klimov et al., 1995; Weinstock et al., 2003), during which time drug pressure may selectively enrich oseltamivir-resistant viruses. This was the case for the two patients examined in this study, both of whom shed the virus for more than two weeks and were admitted to the ICU. The early detection of low levels of oseltamivir-resistant mutants in specimens from such patients would allow clinicians to make alternative treatment decisions (WER, 2009) and select the most appropriate anti-viral drug to use.

The low frequency of H1N1pdm oseltamivir-resistant viruses observed to date is in stark contrast to the situation with seasonal H1N1 viruses during 2007–2008, when, after previously rare occurrences, an H275Y strain appeared in Europe and within 12 months had spread globally (Hurt et al., 2009a; Lackenby et al., 2008b). One possible explanation is that, unlike the seasonal H1N1 H275Y mutant, the H1N1pdm oseltamivir-resistant mutation compromises viral fitness. It is possible however, that further mutations within the H1N1pdm virus genome may enable

**Table 4**
Nucleotide differences in HA and NA genes of viruses in pre- and post-treatment specimens from Patients 1 and 2.

| Patient | Specimen | Nucleotide position[a]/changes | | | | | GenBank Accession No. | |
|---|---|---|---|---|---|---|---|---|
| | | HA | | | NA | | HA | NA |
| | | 243 | 408 | 1320 | 231 | 823[b] | | |
| Patient 1 | Pre-treatment | C | A | A | G | C | HM754655 | HM624082 |
| | Post-treatment | C | A | A | G | T | HM624086 | HM624083 |
| Patient 2 | Pre-treatment | T | G | C | A | C | HM624085 | HM624081 |
| | Post-treatment | T | G | C | A | C & T | HM624087 | HM624084 |

[a] Nucleotide positions in both HA and NA sequences are counted from the first ATG. All the mutations listed were synonymous mutations except at the H275Y position.
[b] The position of the H275Y mutation.

H275Y mutant viruses to maintain fitness and readily transmit oseltamivir-resistant viruses throughout the community (Bloom et al., 2010).

The NA inhibition assay has traditionally been used to monitor influenza antiviral drug resistance, because it can identify any neuraminidase resistant viruses regardless of the mutation. Currently, however, the H275Y mutation in the N1 of H1N1pdm viruses (although relatively rare) is by far the major mutation responsible for oseltamivir drug resistance. This makes pyrosequencing the ideal method for monitoring this resistance mutation in H1N1pdm viruses because it is sensitive, accurate, allows high throughput, and most importantly it is capable of monitoring viral mixtures for any mutations in the test region in clinical samples or influenza virus isolates from patients.

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