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Detection of the rapid emergence of the H275Y mutation associated with oseltamivir resistance in severe pandemic influenza virus A/H1N1 09 infections

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ABSTRACT

In 2009 a new swine-origin influenza virus A/H1N1 (A/H1N1 09) emerged, causing the century's first pandemic. Most isolates of the new A/H1N1 09 virus are susceptible to neuraminidase inhibitors, but the H275Y mutation in the neuraminidase gene region associated with high-level oseltamivir resistance has been detected. Using rolling circle amplification (RCA) technology, 96 A/H1N1 09-specific RT-PCR positive clinical samples collected from 80 oseltamivir-treated and untreated patients were screened for the presence of the H275Y mutation. Samples positive for 275Y mutation by RCA were cloned and sequenced for confirmation. From 25 patients who had been treated with oseltamivir and remained A/H1N1 09 RT-PCR positive, we identified three (12%) individuals with the H275Y mutation: one immuno-suppressed adult, one immuno-competent adult and one child. Samples collected at multiple time points from the two adults showed a switch from wild-type oseltamivir-sensitive 275H to oseltamivir-resistant 275Y population after 9 days of treatment. The child had the 275Y mutation detected after being persistently A/H1N1 09 RT-PCR positive while receiving oseltamivir treatment. Resistance was not detected in 17 pre-treatment samples and 54 A/H1N1 09 RT-PCR positive outpatients. RCA demonstrates the rapid emergence of the H275Y resistance mutation in individuals with severe A/H1N1 09 infection receiving neuraminidase inhibitors. Rapid detection of oseltamivir resistance in severe infection is essential for patients to receive maximum therapeutic benefit. In the light of emerging resistance, close monitoring and understanding of the nature and dynamics of resistance mutations in newly emerging strains should be a priority.

1. Introduction

With the emergence of new swine-origin influenza virus A/H1N1 in 2009, strategies aimed at containing the spread and easing the impact of this virus have been implemented (Dawood et al., 2009). These include use of oseltamivir, recommended by the World Health Organization and the Centers for Disease Control and Prevention for the treatment of people who are either high risk or who have persistent or rapidly worsening symptoms.

The neuraminidase inhibitors oseltamivir and zanamivir reduce the duration of the symptoms of seasonal influenza infection when started early after disease onset; when administered as prophylaxis, they effectively prevent clinical infection (Hayden et al., 1999; Kim et al., 1997). Resistance to the neuraminidase inhibitors (in particular oseltamivir) following treatment of seasonal influenza was initially thought to be uncommon (Hurt et al., 2003; Monto et al., 2006), until described in children (Kiso et al., 2004) and immuno-suppressed hosts (Ison et al., 2006). Of major impact has been the emergence and spread in late 2007 of high-level oseltamivir-resistant seasonal influenza A (H1N1) viruses, characterized by a mutation of histidine to tyrosine at residue 275 of the NA gene (Besselaar et al., 2008; Sheu et al., 2008). In addition, a similar H275Y mutation has been associated with clinical failure in oseltamivir treatment of influenza A/H5N1 zoonotic infections (de Jong et al., 2005).

The widespread use of neuraminidase inhibitors for pandemic control may create increasing selective pressure for the emergence and spread of drug-resistant influenza. Therefore, there is a

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need to understand trends in the susceptibility of circulating pandemic influenza strains to antiviral agents, and to provide rapid resistance testing in patients where antiviral treatment may be failing. To date, most reported resistance is in retrospective surveillance studies; the lack of clinically relevant testing has been due to a dependence on cell-culture methods (McKimm-Breschkin et al., 2003). Recently, we have developed a padlock probe based assay for high throughput and rapid resistance testing performed directly on clinical samples (Steain et al., 2009). Together with other approaches (Deyde and Gubareva, 2009; Lackenby et al., 2008), genotyping methods offer accurate, rapid and clinically relevant resistance testing while also providing information in understanding the emergence of resistance and preventing its early spread.

In this study, we used padlock probes in conjunction with rolling circle amplification (RCA) directly on clinical samples collected from A/H1N1 09-infected individuals to monitor the emergence and spread of oseltamivir resistance during the recent pandemic wave in Australia. A padlock probe comprises of two sequences complementary to the 5′ and 3′ termini of the target sequence joined by a genetic linker region. When they hybridize, head to tail, to the target, the 5′ and 3′ ends of the probe are juxtaposed. Mediated by DNA ligase with high allele discrimination ability (Gerry et al., 1999), the juxtaposed 5′ and 3′ ends of the probe form a closed, circular molecule (Nilsson et al., 1994). The formation of circular molecule is highly sequence dependant as a single base mismatch at either ends of the probe (especially 3′ end) to the target template is enough to cause topology-distortion and prevent the ligase from creating the phospho-diester bond. As a consequence the padlock probe will remain as liner molecule (Faruqi et al., 2001). The superiority of padlock probes in accurately identifying SNPs, including drug resistance mutations, has been demonstrated (Faruqi et al., 2001; Wang et al., 2009). In addition, the circularized probe can further serve as a signal, which can be increased exponentially by RCA for sensitive detection (Lizardi et al., 1998; Wang et al., 2009).

2. Materials and methods

2.1. Clinical specimens

A total of 96 samples from 80 individuals, who were influenza A/H1N1 09 RT-PCR positive using an in-house method (Kok et al., 2009), were analyzed. All but one sample were collected from patients admitted to Westmead Hospital, Sydney, Australia between May and July 2009. The additional sample was provided by the Children's Hospital at Westmead, Sydney, Australia in October 2009, from a 1-year-old child that was persistently A/H1N1 09 RT-PCR positive after 6 weeks of oseltamivir treatment. Among those 80 individuals, 26 required intensive care admission due to severe infection. All 26 individuals received oseltamivir treatment at 75 mg twice daily orally (or in ventilated patients 150 mg twice daily via nasogastric tube), with 17 pre- and 25 post-treatment samples available from those individuals. Upper respiratory tract samples were also collected from 54 randomly selected outpatients attending the hospital influenza clinics who were A/H1N1 09 RT-PCR positive. This study was approved by the Sydney West Area Health Service Human Research Ethics Committee (HREC2009/7/4.17(3031)).

2.2. RNA isolation and cDNA synthesis

Viral RNA was extracted from respiratory tract samples using the Qiagen Viral RNA extraction kit (Qiagen, Hilden, Germany), and cDNA was synthesized using Roche First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA) according to the manufacturers' instructions.

Table 1
Padlock probe sequence.

3 Probe name	Sequence
H1N1 swine 274Y RCA probe	5′^{A}P-RTAATTAGGGGCATTCATTTCGGATCATGCTTCTTC GGTGCCCATGAGGTGCGGATAGCTCGCGCAGACAC GATAGTCTAAGGAGCATTCCTCATARTA-3′
H1N1 swine 274H RCA probe	5′^{A}P-RTAATTAGGGGCATTCATTTCGGATCATGCTTCTTC GGTGCCCATCCTAGATCAGACGTTCCTGTCCGCGCA GACACGATAGTCTAGGAGCATTCCTCATARTG-3′
Primer	
RCA Primer 1	ATGGGCACCGAAGAAGCA
RCA Primer 2	CGCGCAGACACGATA

A: The 5′-end of probe, p-indicates phosphorylation. For Broad reactivity of the probes, ambiguous positions were introduced according to sequences submitted to genebank and shown as underline.

2.3. PCR amplification of partial viral neuraminidase gene region

A conventional PCR was used to amplify a ~270 bp fragment from the NA gene region covering amino acid 275 from patient-derived cDNA samples. Reaction conditions were 1 cycle of 95 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and 10 min at 72 °C for final extension. The primers used in the PCR were: forward 5′-GGACCAAGTGATGGACA-3′ (753–769), reverse 5′-CCACAACTGCCTGTCTT-3′ (1027–1011). To avoid contamination, appropriate precautions were taken and relevant controls were performed. PCR products were purified using a Millipore PCR purification plate (Millipore, Billerica, MA, USA) and quantified by spectrophotometer. Purified PCR products then served as template for padlock probes genotyping and DNA sequencing.

2.4. Genotyping of amino acid position 275 by padlock probe and rolling circle amplification

Padlock probes that recognize either NA resistance-specific SNPs and wild-type sequence from A/H1N1 09 were designed as previously described (Steain et al., 2009). To improve probe binding, ambiguous positions were also introduced to allow recognition of all polymorphisms during probe design according to reported wild-type and resistance mutation profiles derived from the GenBank (Table 1). The broad reactivity of the probes was also examined carefully by comparison with globally derived pandemic A/H1N1 09 sequences in GenBank.

Standard 275H wild-type template was derived by cloning of PCR amplicons from a patient infected with wild-type strain, and an oseltamivir-resistance 275Y template was generated by using synthetic long DNA template, as described previously (Steain et al., 2009). Both the wild-type 275H and oseltamivir-resistance 275Y templates were sequenced to confirm the presence of desired mutations. The specificity of padlock probes in recognition of corresponding resistance or wild-type template was carried out by 15 cycle ligation of the probes with 10^{11} copies of corresponding resistance or wild-type template in a total reaction volume of 10 μl containing 2 U of Pfu DNA ligase and 1 pmol of padlock probe. The reaction conditions included 5 min at 94 °C to denature the dsDNA followed by 15 cycles of 94 °C for 30 s and 4 min ligation at 65 °C. The ligation mixture was then subjected to exonucleolysis to remove any unreacted padlock probe and template PCR product in order to reduce subsequent ligation-independent amplification events. The exonuclease treatment was performed in a 20 μl volume by adding 10 U each of exonucleases I and III (New England Biolabs, Ipswich, MA, USA) to the ligation mixture and incubating at 37 °C for 30 min followed by 94 °C for 30 s to inactivate the enzymes (Steain et al., 2009). The amplification of circularized padlock probes was performed in a 50 μl volume by adding 8 U of Bst DNA polymerase

(New England Biolabs, Ipswich, MA, USA), 5 μl reaction buffer, 400 μM dNTP mix, 10 pmol of each RCA primer, 5% DMSO (v/v) and 1× Sybr Green I (Sigma–Aldrich, St Louis, MO, USA) to the ligation mixture. The reaction was carried out at 65 °C for 30 min and the accumulation of dsDNA products was monitored on a Corbett RotorGene 3000 real-time PCR machine (Corbett, Mortlake, NSW, Australia). Testing the sensitivity of the resistance-specific padlock was carried out using 10^{11} copies of standard templates containing 100%, 50%, 10%, 1%, 0.5% and 0% (i.e. wild-type only) resistance template in a background of wild-type template as reaction condition describe above. The positive signals for 275Y mutations were recognized as having signals above the standard containing 0.5% of the resistance template, and positive samples were repeated to confirm the initial result.

2.5. Data validation

Patient respiratory tract samples showing a positive signal for the 275Y mutation by RCA were sequenced to verify the presence of the 275Y mutation. To eliminate possible PCR-related artifacts, a separate PCR to amplify near full-length NA gene region with a different primer pair was performed and these PCR amplicons were sequenced. PCR amplicons derived from multiple time points (pre- and post-treatment) in patients with the 275Y mutation were also cloned into pGEM-T Easy Vector System II (Promega, Madison, WI, USA) and transformed into competent *Escherichia coli* JM109 cells, according to the manufacturer's protocol. The plasmid DNA from positive clones was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Twenty clones from each time point were sequenced to compare viral populations.

3. Results

3.1. Specificity, sensitivity and accuracy testing of padlock probes in the detection of A/H1N1 09 275H and 275Y populations

Both A/H1N1 09 275H and 275Y-specific padlock probes were used to target standard A/H1N1 09 275H and 275Y templates. Fig. 1A shows the results for both probes with standard templates, respectively. After 15 cycles of ligation steps followed by exonucleolysis to remove unreacted padlock probe and template PCR product, the amplified circular probe signals were detected. The signal could only be detected in the matched probe–template mixture, and no signals were observed in the unmatched pair despite differing by only one single nucleotide (Fig. 1A). The absence of signal with the unmatched probe and template clearly demonstrates the highly specific mutation discrimination ability of the padlock probes. After proving the specificity of the padlock probes, the sensitivity of 275Y and 275H probe for the detection of resistance and wild-type population in the mixture was determined. The 10^{11} copies of standard template containing 100%, 50%, 10%, 5%, 1%, 0.5%, and 0% of match template in the background of unmatched template were ligated with the 275Y and 275H probe using 15 cycles. RCA detection of the ligated probe signal indicated that even when the 275Y or 275H template was present at only 0.5%

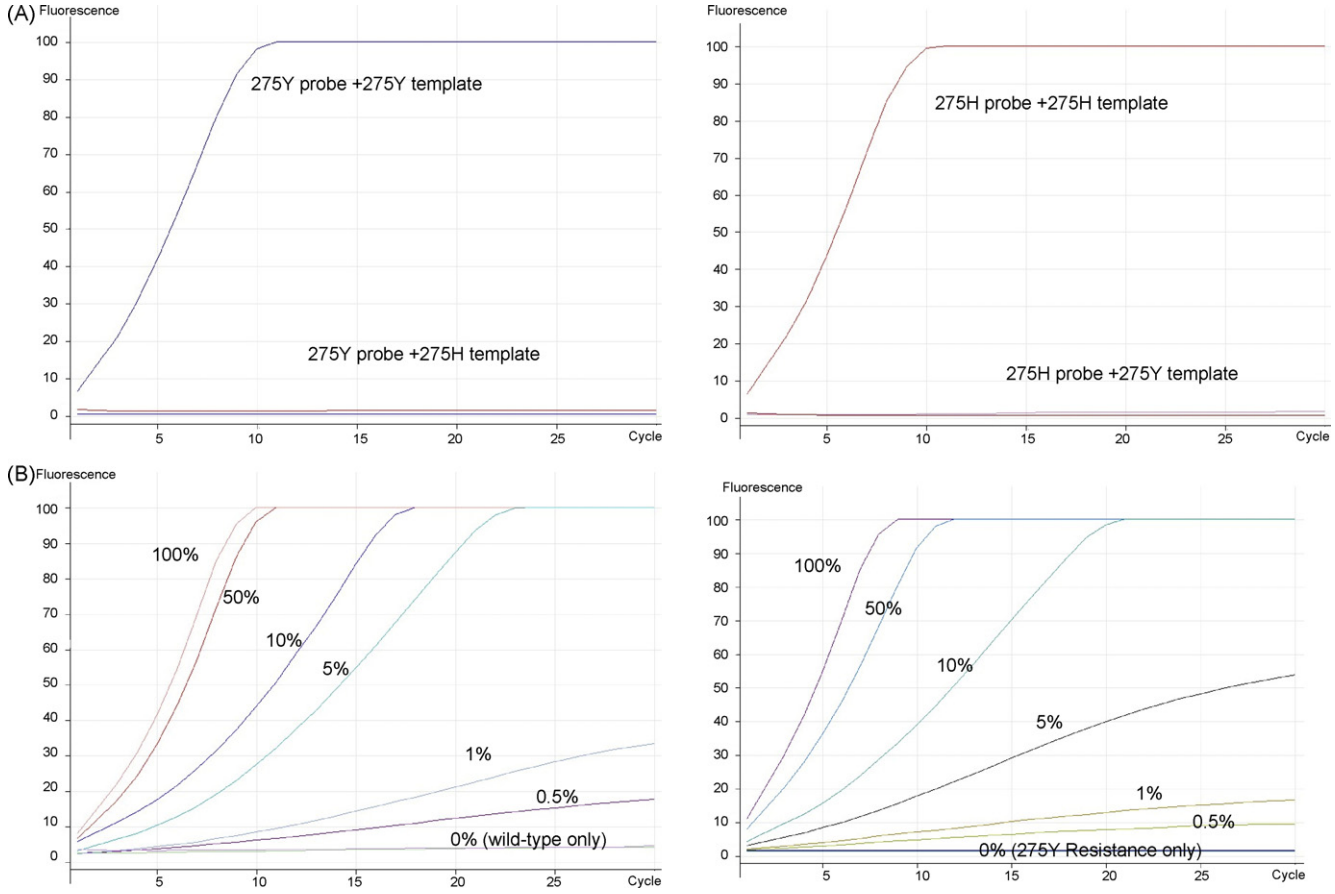

Fig. 1. (A) Specificity testing of the pandemic (H1N1) 2009 275Y (left panel) and 275H (right panel) padlock probe targeting 10^{11} copies of standard 275Y and 275H templates. The ligation reaction was carried out in 15 cycles followed by RCA reaction to amplify circularized padlock probe. Specific probe signals were only detected with matched probe–template pairs shown as significant increasing in fluorescence signal. No signal was detected in unmatched probe–template pairs. (B) Sensitivity testing of 275Y (left panel) and 275H (right panel) padlock probes targeting templates containing various percentages of 275Y and 275H target. Significant elevation of signal was detected in both probes even at the presence of 0.5% of corresponding resistance or wild-type template.

in a background of the other genotype, a positive signal could still be detected (Fig. 1B). The gradual delay in the peak of the signal in response to less copies of template also indicates the possible application of this technique in quantifying detection of resistance population (Fig. 1B). The end product can also be directly visualized by agarose gel electrophoresis and typically the amplified probe showed ladder-like pattern of dsDNA, as shown previously (Steain et al., 2009). The accuracy and reproducibility of both probes were determined by ten individual experimental repeats. Consistent results were observed throughout.

3.2. No evidence of 275Y mutation in untreated patients

From 71 pre-treatment patient samples (54 untreated outpatient samples and 17 samples from 26 hospitalized patients prior to oseltamivir therapy), RCA showed that only wild-type viral populations were detected (Fig. 2A).

3.3. Emergence of 275Y mutation in oseltamivir-treated patients

From 26 hospitalized individuals, 25 patients had samples collected after commencement of oseltamivir. Among these 25 individuals, samples from three patients were identified as 275Y positive by RCA testing of the NA gene PCR amplicons (Fig. 2B), while the other patient samples indicated only wild-type virus. Patient A was a 62-year-old male allogeneic stem cell transplant recipient diagnosed with A/H1N1 09 infection on 7th July 2009 and treated with oseltamivir. He remained A/H1N1 09 RT-PCR positive in samples collected 9 (09-197-3186) and 13 days (09-201-3915) after commencement of oseltamivir. RCA revealed only wild-type virus from the pre-oseltamivir treatment time-point (09-188-2640), but a 275Y-specific positive signal was detected (with no wild-type 275H signal) for the two later time points (Fig. 2B).

Patient B was an immuno-competent 47-year-old female who presented with viral pneumonitis, and had influenza A/H1N1 09 detected on the 14th July from both upper and lower respiratory tract samples (09-195-1618, 09-195-1495). She developed progressive respiratory failure and was admitted to intensive care, ventilated, and received a combination of oral oseltamivir and nebulised zanamivir before improving slowly. Both pre-treatment samples tested negative for 275Y by RCA, with only wild-type viral populations detected (Fig. 2B). Another sample (09-204-2864) was collected 9 days post-oseltamivir therapy and a positive signal was

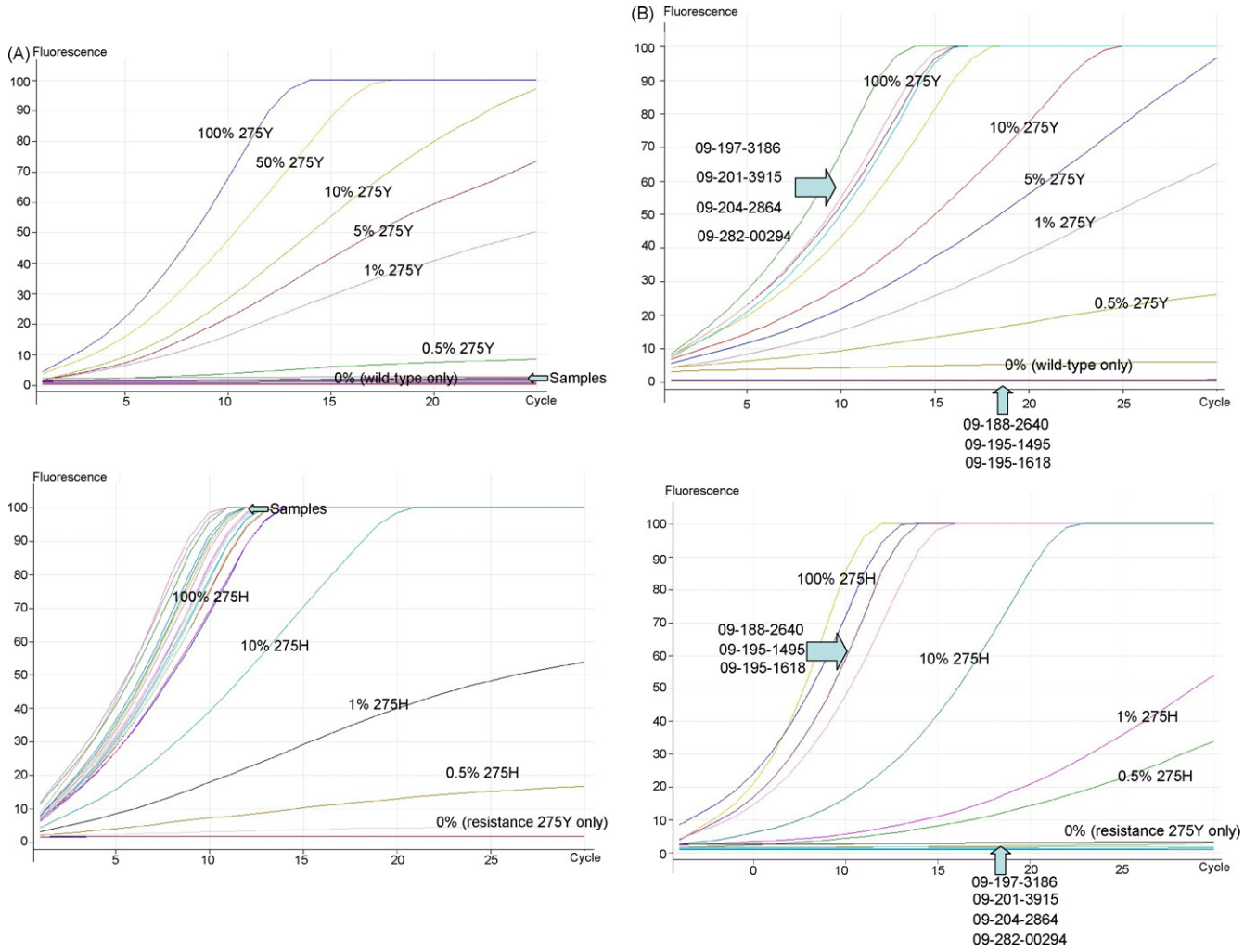

Fig. 2. (A) RCA testing of 54 untreated pandemic (H1N1) 2009-infected outpatients samples together with 17 hospitalised patients derived samples prior to oseltamivir therapy. With 275Y probe, all samples gave negative signals while with 275H probes (upper panel), all samples showed positive signals with pattern similar to 100% 275H control template (lower panel). (B) RCA testing of seven samples from three oseltamivir-treated individuals. Upper panel showed the signal from 275Y padlock probe while the lower panel showed the signal from 275H padlock probe. Four samples (09-197-3186, 09-201-3915, 09-204-2864, 09-282-00294) showed the positive signal for 275Y probes but negative for 275H probes. In contrast, three samples (09-188-2640, 09-195-1495 and 09-195-1618) were negative for 275Y padlock probe but positive for 275H probes.

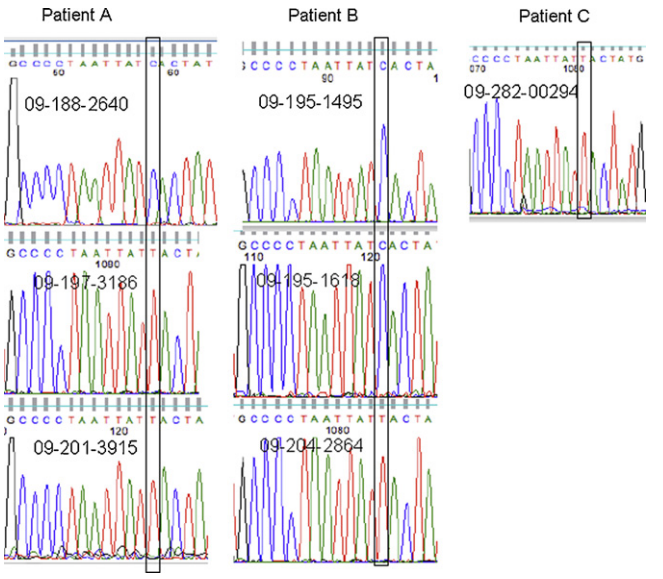

Fig. 3. Sequence chromatogram of pandemic (H1N1) 2009 NA gene region at position 275 from seven samples derived from the three patients. Early samples (09-188-2640, 09-195-1495 and 09-195-1618) from patient A and B showed the wild-type viral population with the presence of C. While post-treatment samples (09-197-3186, 09-201-3915, 09-204-2864, 09-282-00294) showed presence of T. The nucleotide acid was highlighted in the boxed area. Only single post-treatment sample was available for patient C.

detected with the 275Y probe, but no wild-type 275H signal was detected (Fig. 2B).

Patient C was a 19-month-old girl, immuno-suppressed with a malignancy, with persistent positive A/H1N1 09 RT-PCR detected in the respiratory tract in the context of having received multiple courses of single- then double-dose oseltamivir therapy. A single A/H1N1 09 RT-PCR positive sample (09-282-00294) collected 6 weeks after starting oseltamivir treatment was available for RCA, and indicated the presence of 275Y and the absence of wild-type viral populations (Fig. 2B).

Despite the presence of oseltamivir resistance, all patients recovered from their influenza infection. Only patient B received an alternative antiviral agent and was discharged from hospital 32 days after diagnosis. Patient A died of unrelated post-transplant complications 63 days after the diagnosis of influenza.

To validate the RCA results, PCR amplicons for the RCA together with amplicons from a separate PCR that amplifies near full-length NA gene region were sequenced. Sequencing of seven time points from these three patients further confirmed the RCA data. The chromatogram clearly shows the wild-type 275H (CAC) population at the first time point (09-188-2640) of patient A, with 275Y (TAC) observed at the following two time points, 9 days (09-197-3186) and 13 days (09-201-3915) post-oseltamivir therapy (Fig. 3). For the patient B, two samples (09-195-1618, 09-195-1495) collected from the upper and lower respiratory tracts on the first day after hospitalization showed wild-type 275H (CAC) sequences, while the third sample (09-204-2864) collected 9 days post-oseltamivir therapy indicated that the 275Y (TAC) mutation had developed (Fig. 3). The single sample derived from patient C revealed the 275Y mutation (Fig. 3).

PCR products derived from the three individuals were also cloned to analyze viral populations. Analysis of 20 clones from each of the seven time points indicated identical results to RCA. The first time point (09-188-2640) of patient A, and two samples (09-195-1618, 09-195-1495) collected from upper and lower respiratory tract on the first day after hospitalization for the patient B, showed wild-type 275H (CAC) only sequences while the remaining four time points showed only 275Y-containing populations. Taken together, the sequencing, cloning and RCA provided an unambiguous validation of the presence of the oseltamivir-resistance mutation 275Y.

4. Discussion

The neuraminidase inhibitors (oseltamivir and zanamivir) are the drugs of choice for treating A/H1N1 09 pandemic infections, especially as A/H1N1 09 is resistant to the ion channel inhibitors amantadine and rimantidine (Deyde et al., 2010). However, given that the current seasonal influenza A/H1N1 subtype is almost completely resistant to oseltamivir, there is concern that the pandemic A/H1N1 09 virus could also develop resistance to one or both neuraminidase inhibitors. In fact, systematic surveillance conducted by the WHO Global Influenza Surveillance Network has already detected 109 A/H1N1 09 isolates (up to 8 December 2009) that are resistant to oseltamivir characterized by the presence of the H275Y mutation (http://www.who.int/csr/disease/swineflu/frequently_asked_questions/swineflu_faq_antivirals/en/index1.html); over the next 3 months the number of oseltamivir-resistant cases increased to 267. Despite these relatively small numbers, the ongoing global spread of A/H1N1 09 accompanied by wider usage of antiviral drugs may further drive selection of drug-resistant strains and their transmission. To date, the frequency and dynamics of resistance development remains unclear, as there is a lack of systematic follow up of patients who have had samples collected after receiving oseltamivir therapy. In this study, we have shown a frequency of 12% (3/25) oseltamivir resistance emerging in immuno-suppressed and immuno-competent patients with severe influenza requiring intensive care admission. The complete switch from wild-type to resistant viral populations within nine days of therapy suggests that resistance develops quickly in this patient population. So far, there is no evidence of community transmission of oseltamivir-resistant pandemic A/H1N1 in samples collected during the 2009 southern hemisphere winter, as analysis of untreated or pre-treatment A/H1N1 09-infected individuals indicated only wild-type viral populations. However, person-to-person transmission of oseltamivir-resistant viruses is likely to occur as the use of antiviral drugs continues to increase. Indeed, person-to-person transmission of oseltamivir-resistant A/H1N1 09 has been reported recently (Le et al., 2010). In addition, if there is co-circulation of pandemic and seasonal influenza strains there is concern that the swapping of genetic elements and resistance mutations through reassortment may occur.

In the light of emerging oseltamivir resistance shown in this study together with reports of person-to-person transmission of oseltamivir-resistant A/H1N1 09 (Le et al., 2010), it is likely that the treatment options will be further limited. To achieve the maximum therapeutic benefit of the neuraminidase inhibitors, close monitoring and understanding of the nature and dynamics of resistance mutations in pandemic and seasonal influenza strains is imperative. To improve detection of resistance mutations, various molecular assays have been developed with different levels of technical complexity and manipulation. Compared with other technologies, padlock probes offer better specificity in resistance mutation detection and RCA of the circularized probe signal in isothermal conditions eliminate complicated thermal cycling conditions required by other forms of PCR. Also, this method can be adapted to high-throughput rapid detection of H275Y in clinical samples, potentially providing clinicians with rapid assistance to determine appropriate antiviral therapy of severe influenza.

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