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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