Frequency of oseltamivir resistance in Sydney, during the Newcastle outbreak of community transmitted oseltamivir-resistant influenza A(H1N1)pdm09 virus, Australia, June to August 2011

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Although oseltamivir-resistant pandemic influenza A(H1N1)pdm09 is uncommon in immunocompetent individuals, a recent report from Newcastle, Australia, showed the first sustained community spread, from June to August 2011, of oseltamivir-resistant influenza A(H1N1)pdm09 virus carrying the H275Y neuraminidase (NA) mutation. To determine the frequency and the extent of this viral variant spread in the nearest major city to Newcastle, we performed a sequence-based genotypic assessment on samples from 143 oseltamivir untreated and 23 oseltamivir post-treatment individuals with influenza collected contemporaneously in Sydney, 120 km southwest of Newcastle. The detection of two of 143 (1.4%) community-derived samples containing H275Y suggests a low prevalence of oseltamivir-resistant influenza A(H1N1)pdm09 virus in the general community and no convincing evidence of spread of the NA H275Y-bearing influenza A(H1N1) pdm09 virus. In oseltamivir treated patients, oseltamivir-resistant influenza A(H1N1)pdm09 virus continue to emerge with three of 23 (13%) post-treatment samples containing the H275Y mutation. The observation of signature mutations and distinct phylogenetic relationship in full-length sequences of haemagglutinin and neuraminidase genes derived from 2011 strains against 2009 strains indicates continued genetic evolution and antigenic drift of the influenza A(H1N1) pdm09 viruses circulating in Australia.

Introduction

Although the world has moved into the post-influenza pandemic period after 2009, local outbreaks and transmission of the pandemic influenza A(H1N1)pdm09 virus remained intense in the southern hemisphere 2011 winter [1].

During the 2009 influenza pandemic, almost all tested influenza A(H1N1)pdm09 viruses remained susceptible to oseltamivir and zanamivir [2], but oseltamivir-resistant variants bearing the H275Y neuraminidase (NA) mutation emerged from individuals receiving prophylaxis, and from immunocompromised patients receiving treatment [3-5]. The frequency of oseltamivir resistance mutations was relatively high in immunocompromised adults and young children when under drug selection pressure, suggesting perhaps a relatively low genetic barrier for NA H275Y to emerge in influenza A(H1N1)pdm09 viruses [5].

Oseltamivir-resistant influenza A(H1N1)pdm09 virus with the NA H275Y mutation may present equivalent viral fitness and transmissibility compared to wild-type viruses in animal models, indicating its potential transmission in the general community (similar to NA H275Y-bearing seasonal influenza A(H1N1) viruses circulating prior to 2009) [6], although others failed to confirm these results, and data derived from animal models may not be directly applicable to humans [7].

Currently, the detection of oseltamivir-resistant influenza A(H1N1)pdm09 virus in untreated individuals in the community remains uncommon (generally less than 1%) and transmission has been documented only in closed settings or where there is close contact with an infected individual [8-10]. However, a recent report of the first sustained community transmission of oseltamivir-resistant influenza A(H1N1)pdm09 viruses (detected in 16% of isolates), in Newcastle, Australia, between June and August 2011 [11], has highlighted the potential of widespread movement of oseltamivir-resistant influenza A(H1N1)pdm09 virus.

The same study also observed the genetically related oseltamivir-resistant influenza A(H1N1)pdm09 virus in Sydney, the largest city and transport hub in Australia, and other areas, suggesting the spread of oseltamivir-resistant influenza A(H1N1)pdm09 virus had occurred [11]. To determine the frequency and the extent of spread, a sequence-based genotypic assessment of influenza A(H1N1)pdm09 viruses circulating at the same time as the Newcastle outbreak was performed in Sydney.

Methods

Patient samples

Respiratory tract samples from 143 oseltamivir treatment-naive individuals infected with influenza A(H1N1) pdm09 virus, detected using an in-house nucleic acid test (NAT) [12] were collected between June and August 2011, which covered the time period during the Newcastle outbreak. For comparison, samples from an additional 23 individuals infected with influenza A(H1N1) pdm09 virus (confirmed on laboratory testing) who had completed a five-day course of oseltamivir during same period were also included. This study was approved by the Sydney West Area Health Service Human Research Ethics Committee (HREC2009/7/4.17(3031)).

Genetic analysis

Viral ribonucleic acid (RNA) was extracted from respiratory tract samples using the Qiagen EZI virus mini kit on the automated EZI Advanced XL instrument (Qiagen, Hilden, Germany). Partial NA gene was amplified using the OneStep RT-PCR system (Qiagen, Hilden, Germany) with primers 5' AGACACTATCAAGAGTTGGAGAAACA 3' and 5' TGTGATTTCACTAGAATCAGG 3' according to the manufacturer's instructions. PCR products were purified and served as template for padlock probe recognition, followed by Rolling Circle Amplification (RCA) of probe signal as previously described [5,13,14].

All samples showing a positive signal for the NA H275Y mutation, together with randomly selected samples with wild-type influenza A(H1N1)pdm09 virus, underwent full-length NA and haemagglutinin (HA) gene amplification with the NA primers (NA Ext: 5' GATAATAACCATTGGTTCGG 3', 5' AAATGGCAACTCAGCACC 3', Int: 5' GGTCTGTATGACAATTGGAAT 3', 5' CACCGTCTGGCCAAGACC 3'), and HA primers (HA Ext: 5' GGCAATACTAGTAGTTCTGCTATAT 3', 5' CATATTCTACACTGTAGAGACCC 3', Int: 5' CTATATACATTTGCAACCG 3' and 5' CCATTAGAGCACATCCAGAAAC 3'). PCR products were purified and sequenced (Applied Biosystems, Foster City, CA, USA).

Chromatograms, together with their sequences, were aligned with the influenza A(H1N1)pdm09 consensus sequence derived from Australian sequences submitted to the National Center for Biotechnology Information (NCBI) Influenza Virus Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/) using Sequencher software (Gene Codes Corporation, Ann Arbor, USA), and were carefully examined at the location where resistance mutations have been reported. Sequences generated in this study were deposited in the GenBank database with the accession numbers: JQ624635–JQ624655. Neuraminidase sequences carrying the NA H275Y mutation are represented by GenBank accession numbers JQ624645–JQ624648 and JQ624650, while their correspondent HA sequences accession numbers are JQ624635–JQ624639. Near

Table

Influenza A(H1N1)pdm09 viral sequences from Newcastle, Australia, 2011, retrieved from Global Initiative on Sharing All Influenza Data, and used for the phylogenetic analysis in this study

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI334766	HA	Australia	2011-07-10	A/NEWCASTLE/132/2011	John Hunter Hospital, Virology Unit, Clinical Microbiology	World Health Organization Collaborating Centre for Reference and Research on Influenza	Deng YM, Iannello P, Caldwell N, Leang SK
EPI334768	HA	Australia	2011-07-11	A/NEWCASTLE/151/2011			
EPI334770	HA	Australia	2011-06-20	A/NEWCASTLE/17/2011			
EPI334772	HA	Australia	2011-05-31	A/NEWCASTLE/2/2011			
EPI334780	HA	Australia	2011-07-01	A/NEWCASTLE/82/2011			
EPI334782	HA	Australia	2011-07-04	A/NEWCASTLE/85/2011			
EPI334765	NA	Australia	2011-07-11	A/NEWCASTLE/151/2011			
EPI334767	NA	Australia	2011-07-10	A/NEWCASTLE/132/2011			
EPI334769	NA	Australia	2011-07-01	A/NEWCASTLE/82/2011			
EPI334773	NA	Australia	2011-06-23	A/NEWCASTLE/37/2011			
EPI334781	NA	Australia	2011-07-04	A/NEWCASTLE/85/2011			
EPI334783	NA	Australia	2011-07-04	A/NEWCASTLE/89/2011			

GISAID: Global Initiative on Sharing All Influenza Data.

We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based. All submitters of data may be contacted directly via the GISAID website: www.gisaid.org.

full-length NA and HA gene alignment was carried out by using the Clustal W program available from biomanager (https://biomanager.info/); Amino acids were numbered starting after the signal peptide (DTLC) and/or the first methionine. Phylogenetic trees were constructed using the neighbour-joining distance matrix algorithm with the Kimura 2 parameter as an evolutionary model and tested using the bootstrap method with 100 replicates. The trees were rooted with the reference strain A/California/7/2009 HA (GenBank accession number: FJ969540) and NA (GenBank accession number: FJ984386) sequences respectively, representing earlier pandemic viral sequences. Sequences from 2009 influenza A(H1N1)pdm09 viruses for HA (GenBank accession numbers: CY055700, CY055756, CY055534, CY055558 and GQ160611) and NA (GenBank accession numbers: CY055798, CY055544, CY055910, CY055678 and CY055567) from Australia, together with 2011 influenza A(H1N1)pdm09 isolates from Australia and other parts of the world (GenBank accession numbers for HA: JN561789, CY099996, CY092864, CY092094, JN714527, CY092856, CY089463, and NA: JN716363, CY092866, JN714545, CY092858, CY089465, CY111288) were retrieved from the NCBI Influenza Virus Sequence Database and six NA H275Y-bearing strains derived from Newcastle, Australia were also included for comparison (Global Initiative on Sharing All Influenza Data (GISAID) (www.gisaid.org) with accession numbers: HA: EPI334766, EPI334768, EPI334770, EPI334772, EPI334780, EPI334782 and NA: EPI334765, EPI334767, EPI334769, EPI334773, EPI334781, EPI334783).

Results

Two of the 143 (1.4%) individuals who had not been treated with oseltamivir had viruses containing the NA H275Y mutation, as did three of 23 (13%) individuals post-treatment with oseltamivir. Statistical analyses of the difference in the frequency of oseltamivir-resistance between treated and untreated patients, by chi-squared test, indicated that the frequency of oseltamivir-resistance was significantly higher ($P<0.001$) in treated patients.

Full-length NA gene sequencing of two community-derived (GenBank accession number JQ624645 and JQ624646) and three post-treatment influenza samples (GenBank accession number JQ624647, JQ624648 and JQ624650) showing positive signal for the NA 275Y probe further confirmed the presence of the NA H275Y mutation in viruses that infected these five individuals. A comparison of full-length HA and NA sequences derived from influenza A(H1N1)pdm09 viruses carrying NA H275Y mutation and five randomly selected wild-type influenza A(H1N1)pdm09 viruses collected during the same time period in Sydney showed closely related virus (99.65–100% HA nucleotide similarity and 99.22–100% NA nucleotide similarity), although two additional NA amino acid substitutions, NA V83A, and NA E128G, were observed from two distinct strains (GenBank accession number: JQ624645 and JQ624650) (Figure 1A) in the NA H275Y-bearing influenza A(H1N1)

Figure 1

Alignments of haemagglutinin (n=10) and neuraminidase (n=10) amino acid sequences from influenza A(H1N1) pdm09 viruses in Sydney, Australia, in 2011, to respective haemagglutinin and neuraminidase consensus sequences from influenza A(H1N1)pdm09 viruses in Australia in 2009

A. Neuraminidase amino acid sequence alignment

Amino acid number[a]	44	62	83	128	241	275	369	386
CON_NA2009:	N	V	V	E	V	H	N	N
JQ624645:	S	I	A	.	I	Y	K	S
JQ624646:	S	I	.	.	I	Y	K	S
JQ624647:	S	I	.	.	I	Y	K	S
JQ624648:	S	I	.	.	I	Y	K	.
JQ624650:	S	I	.	G	I	Y	K	S
JQ624649:	S	I	.	.	I	.	K	S
JQ624651:	S	I	.	.	I	.	K	S
JQ624652:	S	I	.	.	I	.	K	S
JQ624653:	S	I	.	.	I	.	K	S
JQ624654:	.	.	.	.	I	.	K	.

B. Haemagglutinin amino acid sequence alignment

Amino acid number[a]	129	154	160	202	214	391	468
Antigenic site number[b]	112	137	143	185	197	374	451
CON_HA2009:	E	P	S	S	A	E	S
JQ624635:	K	.	G	T	T	K	N
JQ624636:	.	.	G	T	T	K	N
JQ624637:	K	H	G	T	T	K	N
JQ624638:	K	.	G	T	T	K	N
JQ624639:	K	.	G	T	T	K	N
JQ624641:	K	.	G	T	T	K	N
JQ624642:	K	.	G	T	T	K	N
JQ624643:	K	.	G	T	T	K	N
JQ624644:	.	.	G	T	T	K	N
JQ624655:	K	.	G	T	T	K	N

CON_HA2009: consensus haemagglutinin amino acid sequence of influenza A(H1N1)pdm09 viruses in 2009; CON_NA2009: consensus neuraminidase amino acid sequence of influenza A(H1N1)pdm09 viruses in 2009.

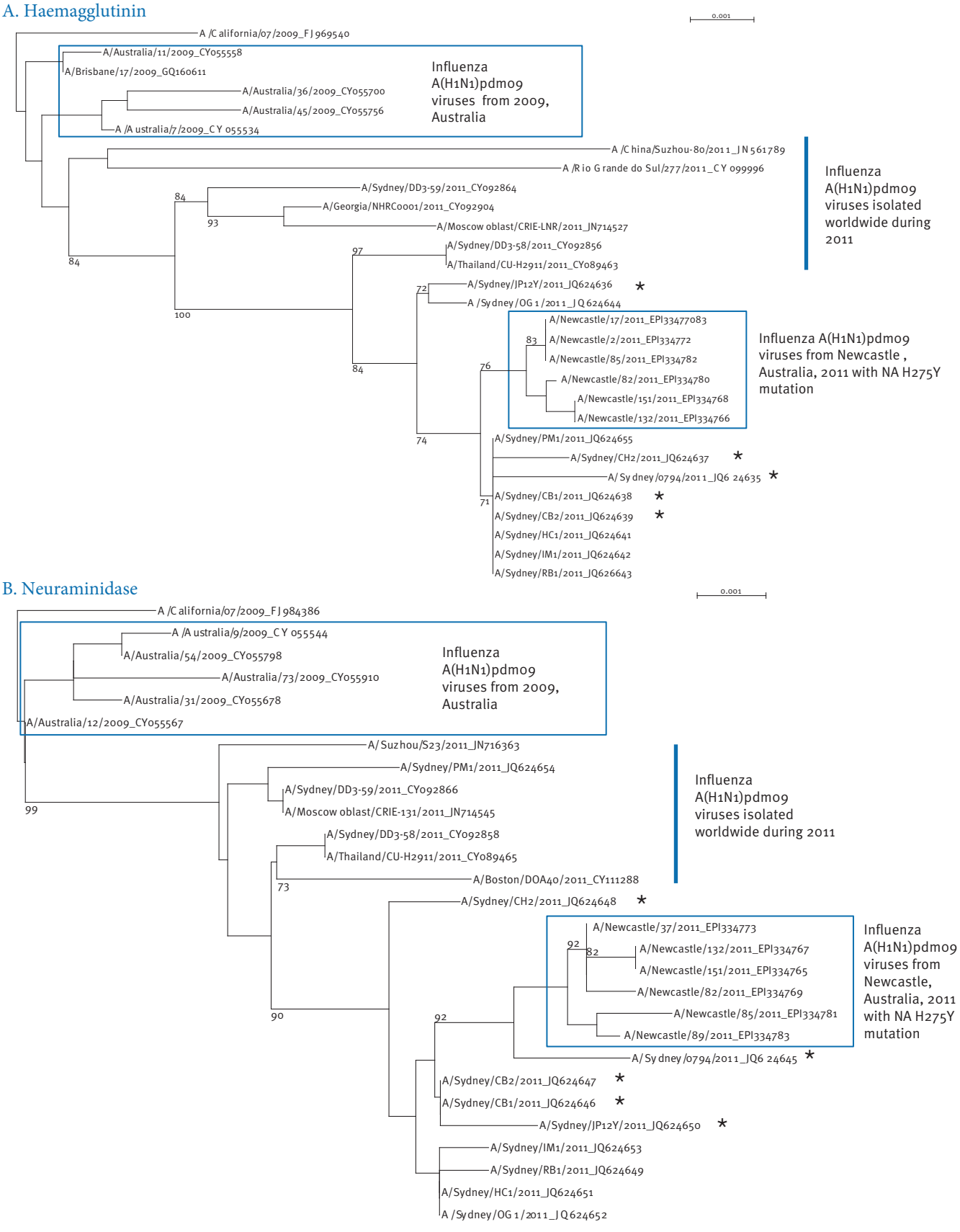
In each of the alignments in panel A and B, a dot indicates an amino acid identical to the respective 2009 consensus sequence. Except for the consensus sequences, the Genbank database accession number figures for each sequence.

[a] The amino acid numbering starts at the first methionine.

[b] The antigenic site amino acid numbering starts after the signal peptide.

Figure 2

Phylogenetic analysis of (A) haemagglutinin and (B) neuraminidase genes nucleotide sequences from influenza A(H1N1) pdm09 viruses isolated in Sydney, Australia, 2011



GISAID: Global Initiative on Sharing All Influenza Data; NA: Neuraminidase.

Nucleotide sequences from influenza A(H1N1)pdm09 viruses isolated in Sydney, Australia, 2011 were compared against influenza A(H1N1)pdm09 viruses sequences from Australia and other parts of the world. GenBank or GISAID accession numbers are shown. Bootstrap values figure on the branches adjacent to the tree nodes. The 2011 influenza A(H1N1)pdm09 virus sequences from GenBank are highlighted by vertical lines. Sequences derived from viruses isolated in Sydney bearing the NA H275N mutation are indicated by an asterisk.

The authors gratefully acknowledge the originating and submitting laboratories who contributed sequences used in the phylogenetic analysis to GISAID.

pdm09 virus. When compared with influenza A(H1N1) pdm09 sequences derived in 2009 during the first pandemic wave in Australia, the 2011 sequences showed five additional mutations (N44S, V62I, V241I, N369K and N386S) in NA and six additional amino acid substitutions (E130K, S160G, S202T, A214T, E391K and S468N) in HA (Figure 1). These were commonly present in most of the 2011 sequences and independent of their resistance profile.

Phylogenetic analyses based on the full-length NA and HA genes confirmed our observation that most of the circulating influenza A(H1N1)pdm09 viruses during the 2011 season in Sydney were distinct from those collected during the 2009 season (Figure 2). The circulating influenza A(H1N1)pdm09 viruses during the 2011 season in Sydney were also clearly associated with influenza A(H1N1)pdm09 viruses collected from Newcastle, Australia at the same period when the outbreak of community transmitted oseltamivir-resistant influenza A(H1N1)pdm09 virus had occurred. By including six full-length HA and NA sequences characterised by NA H275Y mutation from Newcastle [11] (Figure 2), a close phylogenetic relationship of viruses was observed between Sydney and Newcastle. The presence of different influenza A(H1N1)pdm09 viruses in 2011 was also supported by phylogenetic analysis that included influenza A(H1N1)pdm09 viruses isolated worldwide during 2011: these sequences (GenBank accession numbers: JN561789, CY099996, CY092864, CY092940, JN714527, CY092856, CY089463 for HA, and JN716363, CY092866, JN714545, CY092858, CY089465, CY111288 for NA) formed a single cluster with our sequences (Figure 2). Also, a close phylogenetic relationship was observed in both NA and HA of the NA H275Y-bearing oseltamivir-resistant influenza A(H1N1)pdm09 viruses with wild-type variants collected at same time (Figure 2). The presence of NA H275Y-bearing oseltamivir-resistant influenza A(H1N1)pdm09 viruses at various locations in the phylogenetic tree further confirms that the NA H275Y viruses emerged several times in Sydney rather than as a clonal expansion of a single resistant mutant (Figure 2).

Discussion

Influenza A(H1N1)pdm09 strains remained the predominant influenza virus circulating in the southern hemisphere in 2011 [1]. Although oseltamivir resistance amongst influenza A(H1N1)pdm09 viruses worldwide has been low, the recent occurrence in Newcastle, Australia, of the first significant community outbreak of NA H275Y-bearing oseltamivir resistant influenza A(H1N1)pdm09 virus has raised concerns about transmission elsewhere [11]. To determine the frequency and the extent of the spread of these oseltamivir-resistant influenza A(H1N1)pdm09 viruses in Sydney, Australia, the adjacent major city and transport hub, respiratory tract samples collected contemporaneously from influenza NAT positive individuals were examined for the presence of the NA H275Y mutation. Of 166 samples collected from June to August 2011, 1.4% of samples collected from untreated patients and 13% of samples collected after five days of oseltamivir treatment contained the NA H275Y mutation. These rates approximate previous studies of oseltamivir resistance in influenza A(H1N1)pdm09 viruses [3,5,9], although more viral strains would need to be analysed before this conclusion could be confirmed. It is worth noting that the frequency of oseltamivir-resistance is significantly higher ($P<0.001$) for treated rather than untreated patients, confirming that resistance usually emerges in response to antiviral drug selection pressure. As only 1.4% of untreated patients carried oseltamivir resistance, there is no convincing evidence of significant community transmission of NA H275Y-bearing influenza A(H1N1)pdm09 virus within Sydney at the same time or following the Newcastle outbreak. Geography and the high degree of population travel between these two cities highlights that rapid responses and testing of large numbers of viruses is important following the first identification of clusters of resistance to determine if community transmission is occurring.

Genetic characterisation of influenza A(H1N1)pdm09 viral strains derived from Sydney suggested close relatedness of viruses isolated in 2011, regardless of their resistance profile. This relationship, evidenced by sharing of signature changes different to 2009 variants, provides evidence of continued viral evolution as well as suggesting recent emergence and limited spread of oseltamivir-resistant variants. This evolutionary process of influenza A(H1N1)pdm09 virus after its introduction to human population and its impact on the effectiveness of current vaccine remains to be clarified. The presence of additional amino acid substitutions in two of the NA H275Y-bearing strains' NA genes also raise the possibility that these changes may be needed for oseltamivir-resistant influenza A(H1N1) pdm09 virus to sustain its replication and transmissibility. Whether the changes are as important as the NA R222Q and NA V234M substitutions in the pre-2009 oseltamivir-resistant seasonal influenza A(H1N1) viruses that are required for sustained transmissibility remains to be investigated [15]. The close association between NA H275Y-bearing influenza A(H1N1)pdm09 viruses from Sydney and Newcastle support the possibility of further spread of such variants although simultaneous local emergence of such variants cannot be fully excluded. In the current situation, prudent use of the neuraminidase inhibitors remains necessary, as does continued monitoring for drug-resistant influenza viruses.

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