

Oseltamivir-resistant influenza viruses circulating during the first year of the influenza A(H1N1)2009 pandemic in the Asia-Pacific region, March 2009 to March 2010

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During the first year of the influenza A(H1N1)2009 pandemic, unprecedented amounts of the neuraminidase inhibitors, predominantly oseltamivir, were used in economically developed countries for the treatment and prophylaxis of patients prior to the availability of a pandemic vaccine. Due to concerns about the development of resistance, over 1,400 influenza A(H1N1)2009 viruses isolated from the Asia-Pacific region during the first year of the pandemic (March 2009 to March 2010) were analysed by phenotypic and genotypic assays to determine their susceptibility to the neuraminidase inhibitors. Amongst viruses submitted to the World Health Organization Collaborating Centre for Reference and Research in Melbourne, Australia, oseltamivir resistance was detected in 1.3% of influenza A(H1N1)2009 strains from Australia and 3.1% of strains from Singapore, but none was detected in specimens received from other countries in Oceania or south-east Asia, or in east Asia. The overall frequency of oseltamivir resistance in the Asia-Pacific region was 16 of 1,488 (1.1%). No zanamivir-resistant viruses were detected. Of the 16 oseltamivir-resistant isolates detected, nine were from immunocompromised individuals undergoing oseltamivir treatment and three were from immunocompetent individuals undergoing oseltamivir treatment. Importantly, four oseltamivir-resistant strains were from immunocompetent individuals who had not been treated with oseltamivir, demonstrating limited low-level community transmission of oseltamivir-resistant strains. Even with increased use of oseltamivir during the pandemic, the frequency of resistance has been low, with little evidence of community-wide spread of the resistant strains. Nevertheless, prudent use of the neuraminidase inhibitors remains necessary, as does continued monitoring for drug-resistant influenza viruses.

Introduction

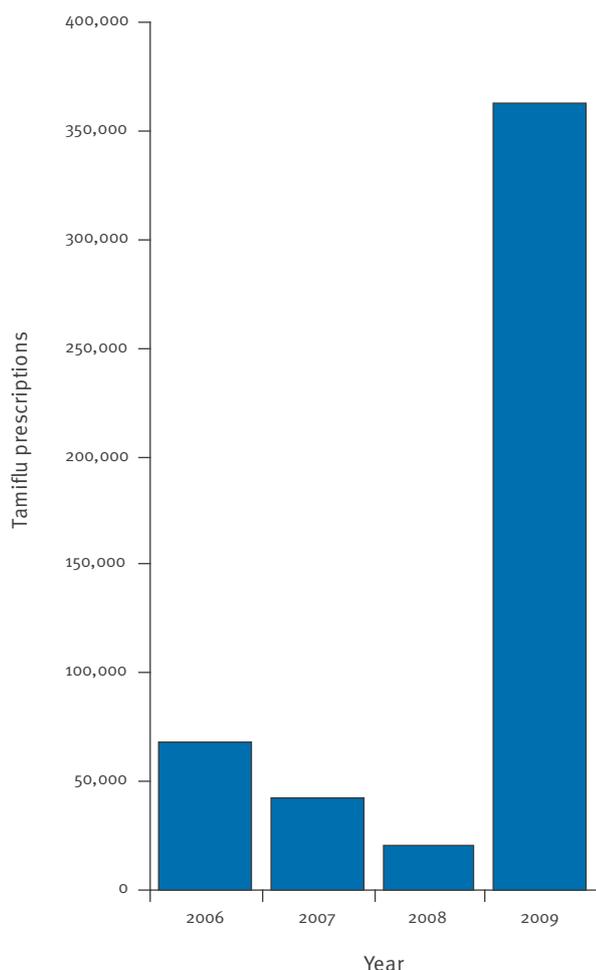
Neuraminidase inhibitors (NAIs) are specifically designed to bind to the conserved neuraminidase (NA) enzymatic site of all influenza A and B viruses, inhibiting the normal function of the enzyme and preventing virus release from the host cell following replication [1]. The NAIs oseltamivir (Tamiflu, Hoffmann-La Roche) and zanamivir (Relenza, GlaxoSmithKline) have been available throughout the world for the treatment and prevention of influenza infections since 1999. Another NAI, peramivir (Biocryst), that has been under investigation as a parenteral formulation, was given emergency use authorisation in some countries such as the United States (US) and Australia during 2009, and in early 2010 was approved for use in Japan for the treatment of both uncomplicated and severe influenza infections [2,3]. In previous years the use of these drugs for the treatment of typical seasonal influenza has been greatest in Japan and the US, but has been very low in other parts of the world such as Australasia, south-east Asia and the South Pacific [4]. Despite their relatively low usage for seasonal influenza and unknown effectiveness against potential pandemic strains, in the last decade many economically developed countries began stockpiling NAIs for use in the event of an influenza pandemic [5,6]. The influenza A(H1N1)2009 pandemic was the first influenza pandemic to have occurred since the NAIs became available.

Early analysis of the pandemic influenza A(H1N1)2009 strain revealed that it was susceptible to the NAIs but was resistant to the adamantanes, an older class of anti-influenza drugs that inhibit the M2 ion channel [7]. In the early months of the pandemic and prior to the production and availability of a specific vaccine, the NAIs were the only specific pharmaceutical intervention

available for the treatment or prevention of infection with this novel strain. In economically developed countries such as Australia, significantly increased amounts of oseltamivir were prescribed during the 2009 pandemic compared to previous years (Figure 1), whereas less economically developed countries in the region used little or no NAIs during the pandemic.

Prior to 2007, only sporadic cases of NAI resistance had been detected, even in Japan and the US where large quantities of the drugs were used. However in late 2007, high frequencies of oseltamivir-resistant seasonal influenza A(H1N1) viruses began to be detected in untreated individuals in Europe and the US [8,9] and by the middle of 2008 these viruses had spread to many parts of the Asia-Pacific region [10]. By 2009 virtually all seasonal influenza A(H1N1) viruses circulating globally were oseltamivir-resistant [11], indicating that the mutant viruses were of equivalent or greater fitness than the previous oseltamivir-sensitive strain,

FIGURE 1
Number of Tamiflu prescriptions filled in Australia between 2006 and 2009



All data derived from IMS Health kindly provided by F. Hoffmann-La Roche Ltd. IMS Rx data represents prescription data, and not necessarily consumption data. Some prescriptions were given based on clinical diagnosis and therefore may include individuals with diseases other than influenza. Data from other countries in the region were not available.

thus dismissing the theory that all viruses with NAI-resistance mutations have a reduced viral fitness [12]. The oseltamivir-resistant seasonal influenza A(H1N1) strains all contained an H275Y mutation in the NA (equivalent to residue 274 based on N2 numbering) [10], a substitution that has previously been detected in other oseltamivir-resistant viruses containing an N1 neuraminidase, such as highly pathogenic influenza A(H5N1) viruses [13]. Therefore, the emergence of the N1-containing 2009 pandemic virus raised concerns that oseltamivir-resistant variants with the H275Y NA mutation (or with other mutations that confer NAI resistance) may emerge and spread throughout the world. Here we report on the frequency of oseltamivir and zanamivir resistance observed in influenza A(H1N1)2009 viruses from the Asia-Pacific region during the first year of the pandemic and describe virological and epidemiological properties of the resistant viruses detected.

Materials and methods

Viruses

Isolates and clinical specimens from Oceania, Asia and Africa were received at the World Health Organization Collaborating Centre for Reference and Research on Influenza (WHO CC), Melbourne, Australia, as part of the WHO Global Influenza Surveillance Network. No recommendations were made regarding the number and type of specimens or isolates sent by submitting laboratories, and the specimens were received from institutes with varying analytical capacity. Some of the samples submitted to the WHO CC may have been biased towards severe or hospitalised cases. Of those confirmed to be the novel influenza A(H1N1)2009 subtype, 1,146 cultured influenza isolates were tested for NAI susceptibility using a functional NA inhibition assay, and a further 342 clinical specimens were tested using molecular techniques for the presence of the H275Y amino acid mutation (Table 1). None of the 342 clinical specimens had a corresponding isolate, therefore each one of the 1,488 samples tested (isolates and clinical specimens) represents an individual patient. All 1,488 samples were taken from patients infected with the influenza A(H1N1)2009 virus within the first year of the pandemic (17 March 2009 to 17 March 2010). The NAI treatment status of patients was not known for the majority of samples received at the WHO CC, although this information was retrospectively obtained for the viruses detected as resistant.

Neuraminidase inhibition assay

All viruses were isolated in Madin-Darby canine kidney (MDCK) cells using standard techniques described previously [14]. Oseltamivir, zanamivir and peramivir susceptibility was measured using a NA inhibition assay that utilises the fluorescent product 4-methylumbelliferone from the substrate 2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) (Sigma, Australia) as a measure of NA activity [15] following a previously published protocol [14]. Oseltamivir carboxylate, the active form of the ethyl ester prodrug oseltamivir

phosphate, was kindly provided by Hoffmann-La Roche Ltd, Switzerland, and zanamivir was kindly provided by GlaxoSmithKline, Australia. Peramivir was kindly provided by BioCryst, Birmingham, US, and was used to test strains with reduced oseltamivir susceptibility. IC₅₀ values (the concentrations required to inhibit 50% of NA activity) were calculated using a logistic curve fit programme 'Robosage' kindly provided by GlaxoSmithKline, UK.

RT-PCR, sequencing and pyrosequencing

The NA and haemagglutinin (HA) genes were amplified by RT-PCR and sequenced using standard techniques [16]. Pyrosequencing followed previously published methods [17] and relative proportions of wild-type and mutant genes were determined using the Pyromark ID v1.0 software following allele quantitation analysis. Neighbour-Joining phylogenetic trees of the HA and NA genes were constructed using the PAUP (V4.0) plugin on Geneious [18,19]. Bootstrap values were calculated from 1,000 NJ replicates. FigTree v1.3.1 was used to display the trees.

Results

Of the 1,146 cell culture-grown influenza A(H1N1)2009 influenza isolates tested for NA susceptibility, nine demonstrated resistance to oseltamivir and none was resistant to zanamivir (Table 1). The mean IC₅₀ ± standard deviation for the fully susceptible influenza A(H1N1)2009 isolates was 0.3 ± 0.2 nM for zanamivir (n=1,146), 0.5 ± 0.4 nM for oseltamivir (n=1,137) and 0.2 ± 0.1 nM for peramivir (n=94). In comparison, the nine oseltamivir-resistant influenza A(H1N1)2009 isolates had mean oseltamivir IC₅₀ values ranging from 279 nM to 462 nM (Table 2), at least 550-fold higher than the mean oseltamivir IC₅₀ value for susceptible wild-type influenza A(H1N1)2009 strains. The oseltamivir-resistant strains remained fully susceptible to zanamivir, but had peramivir IC₅₀ values ranging from 30.6 nM to 42.0 nM, demonstrating an approximate 170-fold increase compared to the mean peramivir IC₅₀ for fully susceptible influenza A(H1N1)2009 isolates (Table 2). Sequence analysis of the oseltamivir-resistant strains revealed that they all contained the H275Y NA mutation.

TABLE 1

Frequency of oseltamivir-resistant influenza A(H1N1)2009 viruses from different countries, Asia-Pacific region, 17 March 2009 to 17 March 2010 (n=1,488)

Region / country	Isolates tested by NA enzyme inhibition assay			Clinical specimens tested by pyrosequencing ^a		Total frequency of oseltamivir resistance
	No. tested	No. oseltamivir-resistant ^b	No. zanamivir-resistant	No. tested	No. with H275Y mutation ^c	
Australasia	808	5	0	312	7	1.1% (12/1,120)
Australia	649	5	0	312	7	1.3% (12/961)
New Zealand	159	0	0	0	-	0
South-east Asia	252	4	0	3	0	1.6% (4/255)
Brunei	12	0	0	0	-	0
Cambodia	10	0	0	0	-	0
Malaysia	64	0	0	0	-	0
Philippines	32	0	0	0	-	0
Singapore	128	4	0	0	-	3.1% (4/128)
Thailand	6	0	0	0	-	0
Other ^d	0	0	0	3	0	0
South Asia and east Asia	24	0	0	0	-	0% (0/24)
Sri Lanka	3	0	0	0	-	0
Macau	21	0	0	0	-	0
South Pacific	62	0	0	27	0	0% (0/89)
Fiji	17	0	0	1	0	0
Guam	5	0	0	5	0	0
New Caledonia	12	0	0	6	0	0
Tahiti	28	0	0	1	0	0
Other ^e	0	-	-	14	0	0
Total	1,146	9	0	342	7	1.1% (16/1488)

NA: neuraminidase.

^a None of the 342 clinical specimens had a corresponding isolate, therefore each one of the 1,488 samples tested (isolates and clinical specimens) represents an individual patient.

^b Viruses were considered resistant if the IC₅₀ exceeded 200 nM. All oseltamivir-resistant strains detected in NA enzyme inhibition assay were confirmed to contain the H275Y mutation.

^c Only includes specimens that contained at least 50% of the H275Y mutation according to allele quantitation pyrosequencing analysis.

^d Papua New Guinea (n=2), East Timor (n=1).

^e Nauru (n=1), Palau (n=1), Kosrae (n=4), Yap (n=3), Chuuk (n=3), Pohnpei (n=2).

Of the nine oseltamivir-resistant H275Y mutant isolates detected in the NA enzyme inhibition assay, five were from Australia and four were from Singapore (Table 1). Pyrosequencing analysis of clinical specimens that could not be cultured (n=342) detected a further seven Australian viruses with the H275Y mutation (Table 1). Apart from these seven strains, an additional five Australian clinical specimens were found to contain the H275Y mutation, but analysis revealed the presence of the mutant virus at a proportion lower than 50% (ranging from 5% to 34 %) and therefore these samples were not included in the count of oseltamivir-resistant strains. In comparison, the seven Australian clinical specimens that were classified as oseltamivir-resistant contained the H275Y mutant at a proportion of 89% to 100% of the viral population.

By combining the data from the functional NA inhibition assay and the pyrosequencing assays, the overall frequency of oseltamivir-resistance in the Australian influenza A(H1N1)2009 viruses submitted to the WHO CC was 1.3% (12/961), while the frequency was slightly higher in the Singaporean influenza A(H1N1)2009 viruses (4/128; 3.1%) (Table 1). As oseltamivir-resistant viruses were not detected among samples from any other countries, the overall frequency of oseltamivir-resistance in influenza A(H1N1)2009 viruses detected in the Asia-Pacific region was 1.1% (16/1,488) (Table 1).

Of the 16 cases in whom oseltamivir resistance was detected, nine patients were considered immunocompromised and were receiving oseltamivir treatment at the time the specimens yielding resistant virus were collected. These patients were ill during the southern hemisphere winter period in the early months of the first pandemic wave and some of them were shedding virus for over three weeks whilst receiving multiple courses of single and double-dose oseltamivir treatment (Table 2). Eight of these patients were undergoing chemotherapy for cancer, including treatment for multiple myeloma (Table 2, Patient 2), prolymphocytic leukaemia (Table 2, Patient 4) and aplastic anaemia (Table 2, Patient 5), as reported in detail previously [20]. One immunosuppressed patient had undergone a renal transplant seven weeks prior to their influenza infection (Table 2, Patient 8). Following infection with an oseltamivir-sensitive influenza A(H1N1)2009 virus, Patient 8 shed both oseltamivir-sensitive and -resistant viruses over a period of nine weeks whilst undergoing 36 days of single- or double-dose oseltamivir treatment together with shorter periods of nebulised and intravenous zanamivir treatment (a full case study on this patient has been reported previously [21]).

Seven patients who had an infection with oseltamivir-resistant virus were otherwise healthy and immunocompetent. Of these seven patients, three were receiving oseltamivir treatment at the time of recovery of resistant virus, including a case from Singapore of an

TABLE 2

Patient and virological details for oseltamivir-resistant H275Y mutant influenza A(H1N1)2009 viruses, Asia-Pacific region, 17 March 2009 to 17 March 2010 (n=16)

Patient details						NAI susceptibility of isolates (mean ± standard deviation)		
Patient number	Location	Immunological status	Oseltamivir treatment	Specimen date	Known duration of shedding	Oseltamivir IC ₅₀ (nM)	Peramivir IC ₅₀ (nM)	Zanamivir IC ₅₀ (nM)
1	Singapore	Competent	Yes	30 May 09	27–30 May 09	374.1 ± 37.3	41.6 ± 12.2	0.3 ± 0.04
2	Melbourne, Australia	Compromised	Yes	25 June 09	16–25 June 09	-	-	-
3	Sydney, Australia	Compromised	Yes	20 July 09	–20 July 09	-	-	-
4	Melbourne, Australia	Compromised	Yes	22 July 09	30 June–22 July 09	-	-	-
5	Melbourne, Australia	Compromised	Yes	24 July 09	20–24 July 09	-	-	-
6	Perth, Australia	Compromised	Yes	28 July 09	Unknown	306.7 ± 21.2	33.3 ± 3.4	0.31 ± 0.03
7	Sydney, Australia	Compromised	Yes	10 Aug 09	20 July–10 Aug 09	279.1 ± 44.9	42.0 ± 11.9	0.25 ± 0.05
8	Perth, Australia	Compromised	Yes	12 Aug 09	24 July–24 Aug 09	296.7 ± 20.0	37.8 ± 3.7	0.28 ± 0.02
9	Singapore	Compromised	Yes	14 Aug 09	3–14 Aug 09	462.3 ± 74.3	32.0 ± 5.3	0.32 ± 0.07
10	Perth, Australia	Competent	Yes	14 Aug 09	9–14 Aug 09	292.6 ± 25.2	32.5 ± 5.6	0.23 ± 0.02
11	Sydney, Australia	Compromised	Yes	18 Aug 09	Unknown	312.5 ± 39.0	32.1 ± 5.0	0.30 ± 0.05
12	Darwin, Australia	Competent	No	29 Dec 09	Unknown	-	-	-
13	Melbourne, Australia ^a	Competent	No	15 Jan 10	Unknown	-	-	-
14	Melbourne, Australia ^a	Competent	No	15 Jan 10	Unknown	-	-	-
15	Singapore	Competent	Yes	21 Jan 10	17 Jan–1 Feb 10	295.5 ± 32.1	29.1 ± 2.1	0.26 ± 0.03
16	Singapore	Competent	No	1 Feb 10	Unknown	378.5 ± 67.0	30.6 ± 3.1	0.31 ± 0.03

NAI: neuraminidase inhibitor; IC₅₀: inhibitory concentration reducing 50% of neuraminidase NA activity).

- indicates that the H275Y mutant virus could not be cultured and therefore no isolate was available for NAI susceptibility analysis.

^a Patients were related.

American patient initially infected in New York (Table 2, Patient 1). This case represents the earliest oseltamivir-resistant influenza A(H1N1)2009 virus reported in this study (30 May 2009). Importantly, four of the immunocompetent patients from whom oseltamivir-resistant virus was recovered were not being treated with oseltamivir or any other influenza antiviral drug and had no known contact with other individuals receiving oseltamivir treatment. Each of these four cases occurred between 29 December 2009 and 1 February 2010, well after the main pandemic periods in Australia (late May to early October 2009) [22] and Singapore (late June to early October 2009) [23].

HA and NA gene sequence analysis was conducted on all of the oseltamivir-resistant viruses that were successfully cultured. Phylogenetic trees drawn from sequences derived from this study showed that oseltamivir-resistant and -sensitive strains were distributed throughout different parts of the tree, with bootstrap values showing less than 50% support for the majority of branches (Figure 2). The low bootstrap values are a result of the lack of divergence in the influenza A(H1N1)2009 viruses since their emergence, and as a consequence the genetic data is neither able to support nor disprove the epidemiological conclusions that these strains arose independently and not as part of an emergent group of related variants.

Discussion

Characterisation of the first influenza A(H1N1)2009 viruses from the pandemic revealed that the strains were resistant to the older class of influenza antivirals, the adamantanes [7], similar to the other swine influenza viruses concurrently circulating in North America [24]. Therefore the NAIs were the only class of influenza antiviral drug available for the treatment and prophylaxis of the novel pandemic strain, and were particularly important before the availability of a specific vaccine. The studies published to date indicate that oseltamivir usage in patients was significantly greater than zanamivir usage during the first year of the pandemic [25-27], and was associated with a lower risk of intensive care admission or death in hospitalised patients if commenced within two days of symptom onset [28].

Although increased amounts of oseltamivir and, to a lesser extent, zanamivir were used during the 2009 influenza A(H1N1) pandemic, only 267 oseltamivir-resistant viruses were reported globally from over 10,000 samples during the first year of the pandemic [29]. In this study, oseltamivir-resistant viruses were detected in Australia and Singapore, but not in samples from the South Pacific, New Zealand, Kenya, south Asia and east Asia, although it is of note that only a relatively small number of viruses were available for testing from the regions where resistance was not detected, and that analysis of a greater number of samples may have revealed a low proportion of resistance. Due to insufficient samples it was not possible

to determine if oseltamivir resistance was more prevalent in children than in adults, as has been reported previously for seasonal influenza [30]. It is most likely that the higher apparent frequency of resistance in Australia and Singapore was a reflection of the amount of oseltamivir used there during the pandemic. The frequency of oseltamivir resistance in Australia (1.3%) and Singapore (3.1%), as determined in this study, was no higher than that reported among oseltamivir-treated adult patients infected with seasonal influenza viruses in clinical trials (1-4%) [31,32] but was higher than that observed in community surveillance studies before 2007 [33-35]. However, care should be taken in drawing conclusions about the frequency of resistance either in treated individuals or in specific patient groups (e.g. immunocompromised) as detailed clinical and epidemiological information was unavailable for the majority of the NAI susceptible cases tested in this study. In addition, it should be noted that samples submitted to the WHO CC (and therefore tested in this study) may be biased towards unusual isolates or hospitalised patients, and therefore the actual frequency of oseltamivir resistance in some countries may be lower than reported here.

Before 2007, there was little evidence of community spread of oseltamivir-resistant viruses and resistant strains in untreated patients were only occasionally detected [16,35], presumably due to impaired viral growth and infectivity of the resistant viruses [36-39]. However the global spread of oseltamivir-resistant seasonal influenza A(H1N1) viruses with the H275Y NA mutation during and after 2008 demonstrated the ability of these resistant strains to replicate and transmit efficiently in the absence of drug selective pressure. It is thought that two permissive mutations in the NA, V234M and R222Q, that occurred in seasonal influenza A(H1N1) viruses shortly before the emergence of the H275Y mutant enabled the virus to tolerate the resistance mutation with no impact on viral fitness [40]. To date, neither of these compensatory mutations have been detected in any influenza A(H1N1)2009 viruses (including those reported in this current study), although the majority of influenza A(H1N1)2009 viruses actually possess N at residue 222 rather than R [41]. Nevertheless, future close monitoring of gene sequences is necessary as these, or other, permissive mutations may enable influenza A(H1N1)2009 H275Y mutant viruses to easily transmit throughout the community. In the current study we identified four patients (Table 2, Patients 12,13,14 and 16) who were shedding oseltamivir-resistant viruses even though they were not undergoing oseltamivir treatment, and all were detected during a period of low influenza activity in the southern hemisphere (December 2009 to February 2010). It is unknown if these patients were infected directly by oseltamivir-treated individuals shedding resistant virus, or whether low level transmission of resistant strains is occurring sporadically in the community. Previous studies have shown that H275Y oseltamivir-resistant influenza A(H1N1)2009 viruses was

transmitted from treated to untreated patients within a hospital in Wales [42], and between close contacts during a train journey in Vietnam [43], but there was no evidence of subsequent transmission to the wider community on either occasion.

Many of the specimens analysed in this study contained a mixed viral population of both oseltamivir-resistant and -sensitive viruses, indicating the need for diagnostic tests to detect small proportions of resistant virus in a mixture. The clinical significance of low-level populations of oseltamivir-resistant virus is uncertain, at least in otherwise healthy individuals. Because most oseltamivir-resistant viruses (including the H275Y mutant) remain fully susceptible to zanamivir, early detection of oseltamivir-resistant viruses in a mixed population can facilitate the use of alternative antivirals such as zanamivir, which have the potential to improve patient outcome.

Although the NAIs have been used in Japan and the US for many years, they have had relatively little use elsewhere. Therefore concern existed that sudden

large-scale use of the NAIs in a pandemic, across many countries around the world, may result in the rapid and widespread selection of resistant viruses. Data collected during the first year of the 2009 influenza A(H1N1) pandemic has demonstrated that this has not occurred, with only 1.1% of strains from the Asia-Pacific region found to be oseltamivir-resistant and no detection of any zanamivir-resistant strains. Nevertheless, prudent use of the NAIs to treat infected individuals is encouraged to avoid selection of resistant viruses, which may in turn acquire the ability to transmit efficiently throughout the community, thereby reducing the available options for antiviral treatment.

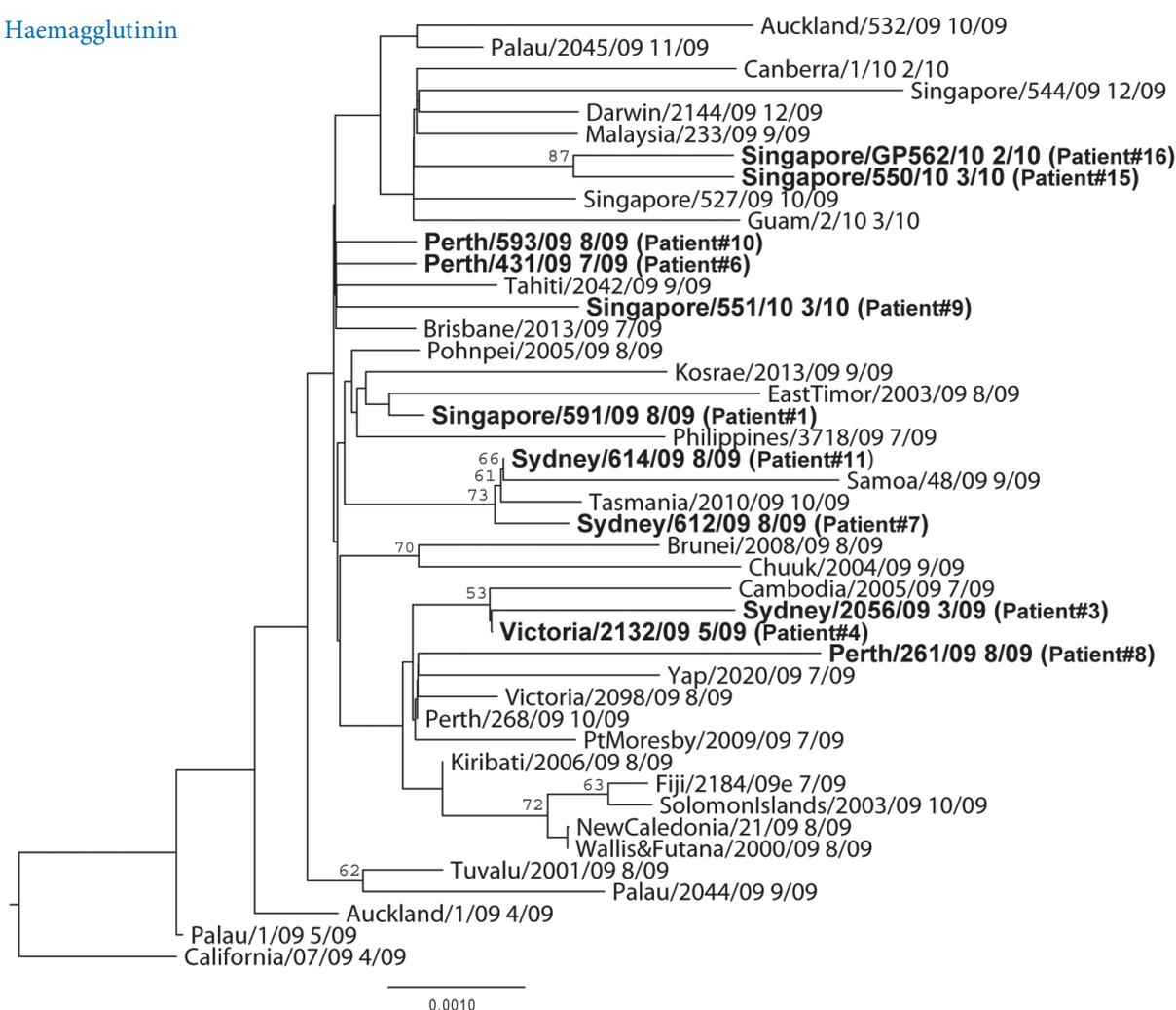
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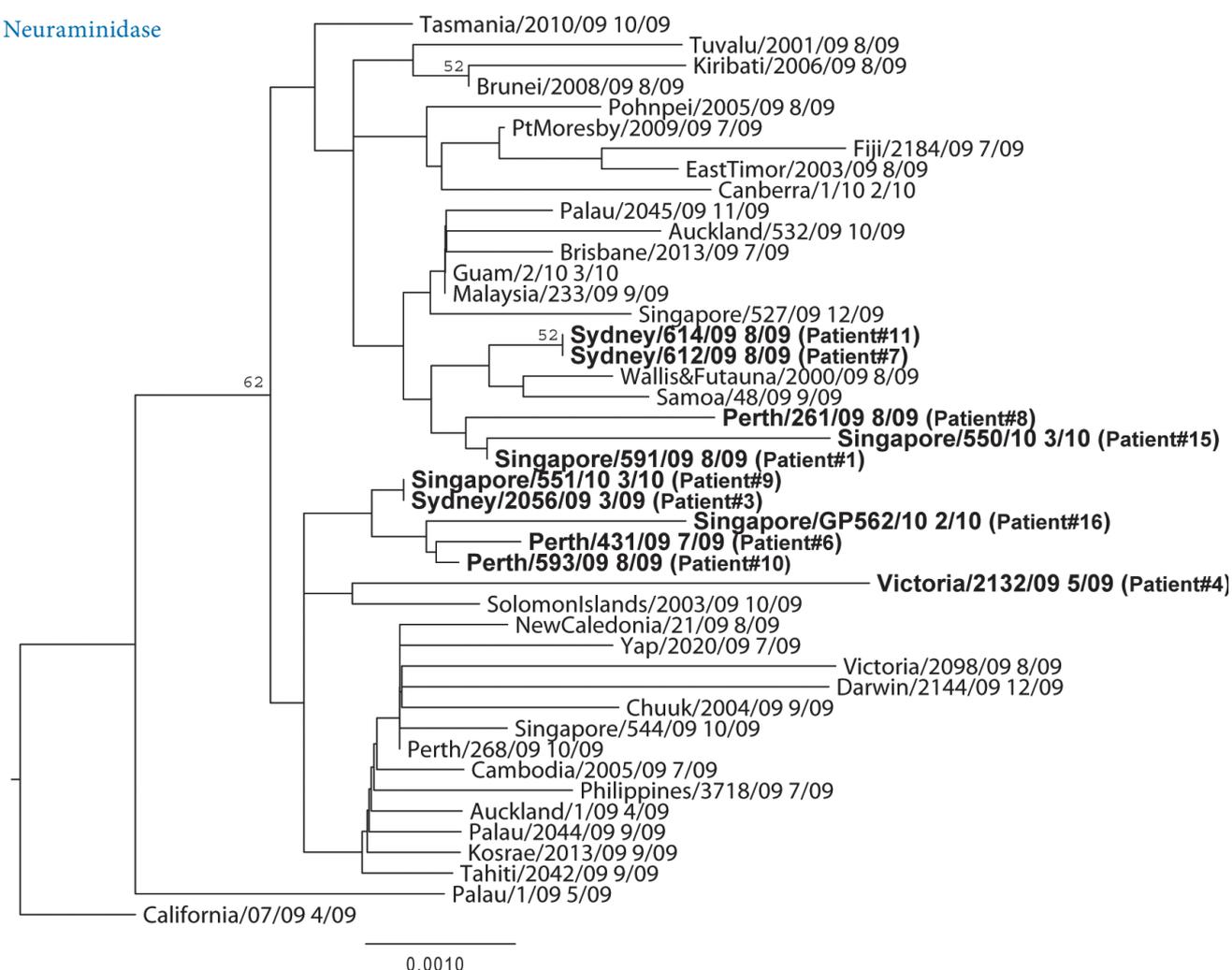
FIGURE 2

Phylogenetic relationships of (A) haemagglutinin and (B) neuraminidase gene sequences for oseltamivir-resistant H275Y mutants and oseltamivir-sensitive influenza A(H1N1)2009 viruses, Asia-Pacific region, 17 March 2009 to 17 March 2010 (n=11 patients)

A. Haemagglutinin



B. Neuraminidase



Full haemagglutinin (HA) and neuraminidase (NA) gene sequences derived from influenza A(H1N1)2009 oseltamivir-resistant H275Y mutant strains (in bold) are compared phylogenetically with oseltamivir-sensitive viruses. Specimen dates (month/year) are included after the strain name. Patient numbers have been included in parentheses after the designation of oseltamivir-resistant viruses to allow cross referencing with case details in Table 2. Culture of virus from Patients 2, 5, 12, 13 and 14 was attempted but was not successful, as such analysis of the original specimen was undertaken but sequence data was not of sufficient quality or length to be included in the phylogenetic trees. Only bootstrap values >50 are shown.

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